Molecular mechanisms of T helper 1 and T helper 2 cell development: differential signalling in response to Interleukin-12

Thesis

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Molecular Mechanisms of T Helper 1 and T Helper 2 Cell Development: Differential Signaling in Response to Interleukin-12

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Abstract

Interleukin-12, a heterodimeric cytokine produced by activated monocytes and dendritic cells, plays a crucial role in regulating IFN-γ production and in the generation of IFN-γ producing T helper 1 cells. Here is shown that the IL-12Rβ2 subunit, a recently cloned binding and signal transducing component of the IL-12R, is expressed on human Th1 but not Th2 clones, and is induced during differentiation of human *naive* T cells along the Th1 but not the Th2 pathway. IL-12 and IFN-α but not IFN-γ induce expression of the IL-12Rβ2 chain during *in vitro* T cell differentiation following antigen receptor triggering, whereas IL-4 inhibits IL-12Rβ2 transcription. Conversely, IFN-γ but not IFN-α can rescue IL-12Rβ2 mRNA expression and restore IL-12 responsiveness in early developing mouse Th2 cells. IFN-α activity in humans is mediated by Stat4.

The DNase Hypersensitive Site analysis allowed the characterisation of chromatin structure and accessibility of the IL-12Rβ2 locus. *Naive* T cells as well as Th2 cells show a relative "closed" chromatin configuration, whereas Th1 cells displayed an accessible chromatin configuration with a complex pattern of DNase Hypersensitivity. The characterisation of the DH sites selectively present in Th1 cells allowed the identification of: *i)* a TATA-less, CpG rich, IL-12Rβ2 minimal promoter *ii)* IL-12 and IFN-α responsive enhancer regions. These regions contain GAS binding sites that not only bind Stat4, but also that are transcriptionally active. These data provide strong evidence that Stat4 plays a key role in the regulation of the Th1-specific expression of IL-12Rβ2. Furthermore, the IL-12Rβ2 GAS sites display a binding selectivity and do not efficiently bind Stat1. Thus, the week effects exerted by IFN-γ in humans on IL-12Rβ2 gene regulation, and the consequent failure to induce Th1 development, can be explained by the inefficient binding of the IFN-γ -induced Stat1 to the IL-12Rβ2 GAS sites.

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1) Introduction

1.1) T helper subsets

In a seminal study a decade ago, Mosmann et al. (Mosmann et al., 1986) showed that murine $CD4^+$ T helper (Th) cells can be classified into two major subsets, termed Th1 and Th2, according to the pattern of lymphokines they produce.

Th1 cells secrete Interferon (IFN)-γ and predominantly promote cell-mediated immune responses, while Th2 cells, which produce IL-4, IL-5 and IL-13, provide help for some B cell responses (Seder and Paul, 1994).

The principal function of Th1 cells is to elicit phagocyte-mediated defense against infection, because Th1 cytokines promote the ability of macrophages to both phagocytose and destroy microbes. Th1 dominant immune responses are often associated with inflammation and tissue injury, because two Th1 cytokines, Tumor Necrosis Factor (TNF)-β and IFN-γ, recruit and activate inflammatory leukocytes (Delayed Type Hypersensitivity reaction (DTH)). Some Th1 cells acquire cytolitic activity and the cytokines produced by Th1 cells promote the differentiation of CD8+ T lymphocytes into active cytotoxic cells (Abbas et al., 1996) (Fig. 1).

IL-4 and IL-5 are the signature cytokines of Th2 cells. IL-4 in particular is the major inducer of B-cell switching to IgE production (Coffman et al., 1993), and therefore plays a crucial role in allergic reactions involving IgE and mast-cells. IL-5 is the principal eosinophil-activating cytokine and mice lacking IL-5 or its receptor are unable to clear helmints (Kopf, 1996) (Fig. 1).

Th cells producing cytokines typical of both Th1 and Th2 clones have been described in both the murine and human system, and they have been named Th0 (Seder and Paul, 1994). The concept of polarized T helper cell subsets was later extended to the human system (Romagnani, 1991), and the balance between Th1 and Th2 subsets was shown to be a major determinant in the outcome of

physiological as well as pathological immune responses, including autoimmune, allergic and infectious diseases (Abbas et al., 1996; Romagnani, 1994) (Tab 1).

The role of various factors influencing the development of T helper cells has been studied using T cell receptor (TCR) transgenic mice as a source of antigen-specific naive $CD4^+$ T cells

Tab I. *Pathological Th1 and Th2 immune responses*

	Host	Response	Comments
Autoimmunity			
Psoriasis vulgaris	H	Th1 response	Th1, but not Th2 cells derived from the psoriasis sites
EAE	M	Th1 cause desease	Th1 clones induce desease anti-IL-12 ameliorates disease
IDDM	M	Th1	
Rheumatoid Artritis	H	Th1	Synovial fluids from RA patients produced IFN-γ ,but not IL-4
Tyroiditis	H	Th1 response	Thelper infliltrating cells secrete Th1 cytokines
Allergy			
Allergy	H	Th2 response	allergen specific clones shows a strong bias towards the Th2 phenotype
Atopic Asthma	H	Th2 response	Th2 cytokines detected in bronchial lavage and in situ studies
Vernal conjunctivitis	H	Th2 response	T- cell clones from conjunctivits produce more IL-4 and less IFN-γ than CTRL cells
Omenn's syndrome	H	Th2 response	immunodeficiency accompanied by eosinophilia, high IGE levels high IL-4 and IL-5

Modified from Mosmann et al, Immunology Today, (1996), 3:138-146

(Hsieh et al., 1993; Kaye et al., 1989; Seder et al., 1992) and with purified naive (Mel-14$^+$) T cells stimulated *in vitro* with plate-bound anti-CD3 antibodies (Schmitt et al., 1994). In the human system, T helper cell development has been studied by analyzing T cell clones

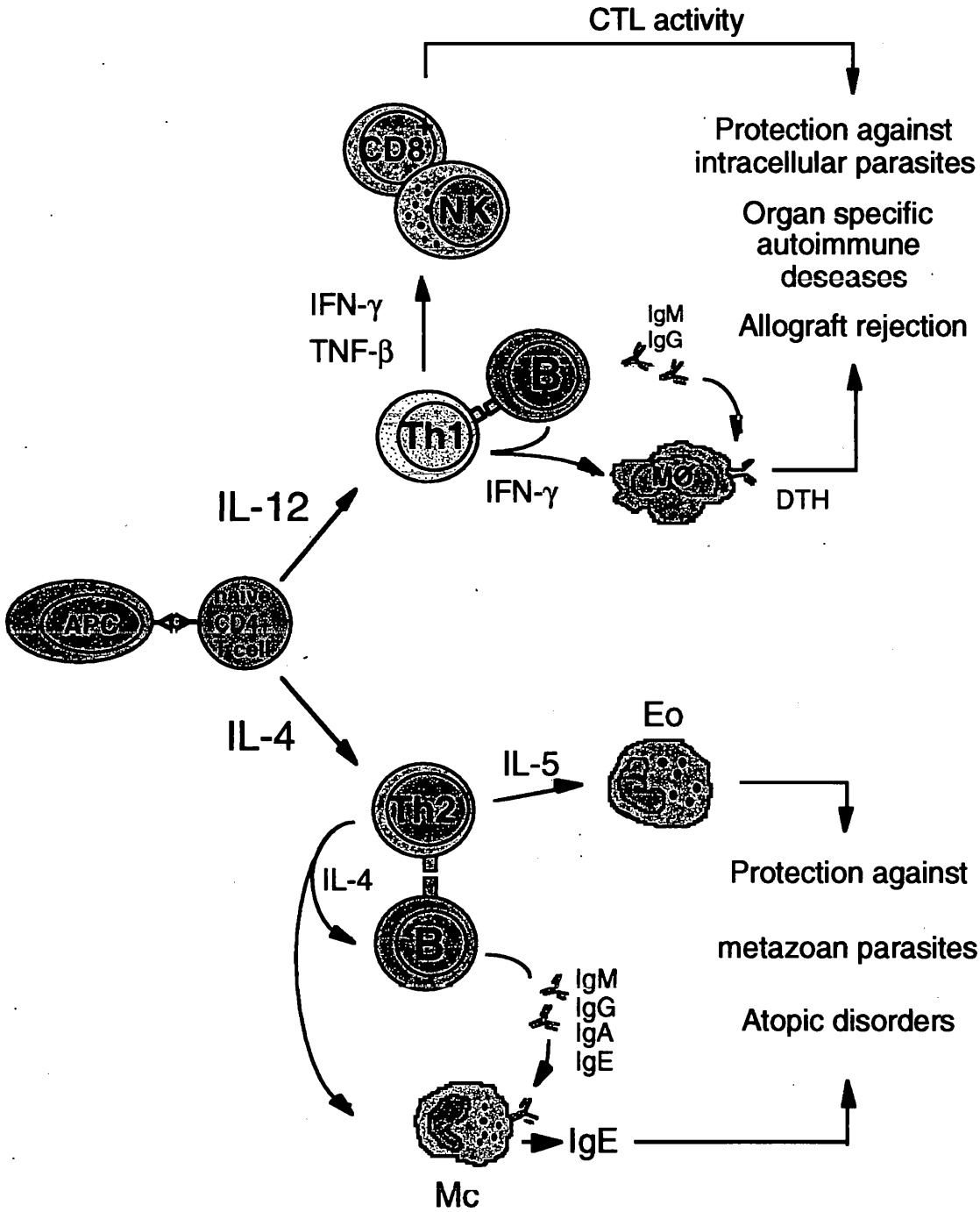

Figure 1. *T helper development. Upper panel*: early production of IL-12 (and IFNs) by cells of the innate immune system promotes the development of Th1 cells, which induces the production of opsonising antibodies and the activation of macrophages (i.e. a DTH reaction). Th1 responses,

together with frequently associated CTL activity, are highly protective against infections caused by intracellular parasites and are responsible for acute allograft rejection, but may result in organ specific autoimmune diseases and other immunopathological disorders. *Lower panel*: early production of IL-4 promotes the development of Th2 cells, which stimulate high antibody production, including IgE, eosinophils and mast cell activity. Th2 responses are protective against metazoan parasites and can be responsible for the development of asthma. (Modified from Romagnani, Immunol. Today, 1997). MO= macrophage; DC=dendritic cell; APC=antigen presenting cell; Mc=Mast cell

obtained either from peripheral blood specific to a variety of bacterial and parasite antigens or allergens, or from target organs of patients with immunopathological disorders (Del Prete et al., 1991; Parronchi et al., 1996; Wierenga et al., 1990). Important results have also been obtained with T cell lines generated by stimulation of cord blood derived neonatal ($CD45RA^+$) T cells (Demeure et al., 1994; Sornasse et al., 1996). A number of studies have shown that the dose and route of antigen administration, the type of antigen presenting cell (APC), and the costimulatory pathways, can influence Th cell differentiation (Bretscher et al., 1992; Constant et al., 1995; Guery et al., 1996; Kuchroo et al., 1995; Lenschow et al., 1996).
However, the cytokines present at the time of priming have a prominent role in inducing polarized Th cell responses (O'Garra, 1998; Seder and Paul, 1994).

1.2) Factors involved in T helper development

1.2.1) Antigen dose

In 1972 Parish and Liew showed that varying the dose of the immunising antigen gives rise to either a humoral or a cell-mediated type of response (Parish and FY, 1972). Several studies were then performed to determine whether Th1- or Th2 responses were elicited by high versus low doses of antigen, all obtaining conflicting results. In most of the studies in which parasites were used as immunogens low doses of antigen induced Th-1 like responses (Bancroft et al., 1994; Bretscher et al., 1992; Parish and FY, 1972). Conversely, when soluble proteins were used instead, low doses of immunogen skewed towards Th2-type

cells (Guery et al., 1996; Wang et al., 1996). These differences observed *in vivo* may be due to the involvement of other factors influencing T helper development, such as the induction of IL-12 production by macrophages after parasite uptake or recruitment of non $CD4^+$ lymphocytes (Constant and Bottomly, 1997). Thus, *in vitro* differentiation systems using TCR transgenic mouse models were utilised to define the effect of antigen dose in T helper development. Bottomly and colleagues reported that high doses of cytocrome-c peptide generated Th2 effector cells, whereas low doses induced Th1 cells (Constant et al., 1995). Using a different TCR transgenic model, O'Garra and colleagues obtained similar results, thus suggesting a direct role of the antigen dose in directing T helper differentiation (Hosken et al., 1995). Nevertheless, the mechanisms underlying these effects are not well understood. An explanation of these findings could be that different antigen concentrations trigger distinct signals from the TCR which influence the differentiation pathway, but still no evidence for this has been documented. Alternatively, high antigen concentrations could lead to repeated T cell stimulation thus reaching a threshold of IL-4 production that would enhance Th2 development (see below).

Furthermore, O'Garra and colleagues also made the important observation that the antigen-dose dependent Th2 development was abrogated by addition of neutralising anti IL-4 antibodies, thus indicating that antigen dose does not seem to be a dominant controller of T helper development (Hosken et al., 1995; O'Garra, 1998).

1.2.2) Costimulation

Since antigen presenting cells (APC) provide precursor Th cells with their first activation signal, the expression of costimulatory molecules on APC and their role in Th differentiation is subject to investigation.

In particular the role of the B7-1 and B7-2 costimulatory molecules investigated in several mouse models gave conflicting results in T helper development (Kuchroo et al., 1995; Lenschow et al., 1996).

In vitro differentiation studies suggested the involvement of B7-2 signalingin Th2 development, while B7-1 failed to influence T cell differentiation (Freeman et al., 1995), these findings where however challenged by other studies (Gause et al., 1997).

Analysis of CD28, the B7 ligand expressed on T cells, supported the requirement of CD28 signaling for development of Th2 cells. CD28 $^{-/-}$ mice infected with lymphocytic choriomeningitis virus showed a marked defect in Th2 dependent antibody responses, yet DTH and cytolytic responses were normal (Lenschow et al., 1996; Shainian et al., 1993). Human naive T cells stimulated with anti-CD3 in the absence of CD28 costimulation produced only IL-2 and IFN-γ, whereas the addition of anti-CD28 mAbs induced both IL-4 and IL-5 (King et al., 1995). The message that can be derived from these studies is the requirement for high levels of costimulation for Th2 development. In this case stronger T cell activation could result in increased IL-4 production by T cells and favor Th2 development. But this is probably an oversimplification and the emerging view tends to emphasise timing and level of costimulation, rather than simply its presence or its absence. For example, costimulation requirements for differentiated Th cells are opposite, since Th1 cells activity is costimulation dependent while Th2 cells do not seem to require any more costimulation (Constant and Bottomly, 1997). Furthermore, there is no direct evidence suggesting a role of CTLA-4 in T helper cell differentiation. CTLA-4 is the other identified receptor of B7 molecules, expressed on T cells. CTLA-4 $^{-/-}$ mice show a severe lymphoproliferative phenotype, suggesting a

general negative regulatory role of this receptor, rather than a role in T helper development (Alegre et al., 1998; Tivol et al., 1995). A possible explanation for the observation that CTLA-4 as well as for B7-1 appear not to be correlated to Th development, may be their delayed upregulation or that their major role is to downregulate ongoing immune responses rather than initiate new ones (Bluestone, 1995).

1.2.3) Genetic background

Different murine genetic backgrounds are known to significantly alter the direction of Th subset development, resulting in resistance or susceptibility to pathogens (Güler et al., 1996; Hsieh et al., 1995; Reiner and Locksley, 1995). BALB/c, BALB/b and BALB/k mice, were described as Th2 prone, while B10.D2 and C57B1/6 are Th2-averse, and DBA/2 usually show an intermediate phenotype (Guery et al., 1996; Reiner and Locksley, 1995). Very little is known about the factors responsible for the genetic predisposition to develop a Th1 versus Th2 type of response, but the ones described so far mainly correlate with cytokine genes. For example the BALB/c default Th2 pathway has been attributed to an early IL-4 production when compared to resistant strains (Launois et al., 1995). Recently, it was observed that B10.D2 exhibit an intrinsically greater capacity to maintain IL-12 responsiveness under neutral conditions in vitro as compared to BALB/c T cells, allowing for longer capacity to undergo IL-12 induced development (Güler et al., 1996; Güler et al., 1997). Analysis of intercrosses between BALB/c and B10.D2 mice identified a locus on murine chromosome 11 that controls the maintenance of IL-12 responsiveness (Gorham et al., 1996) (Fig. 2).

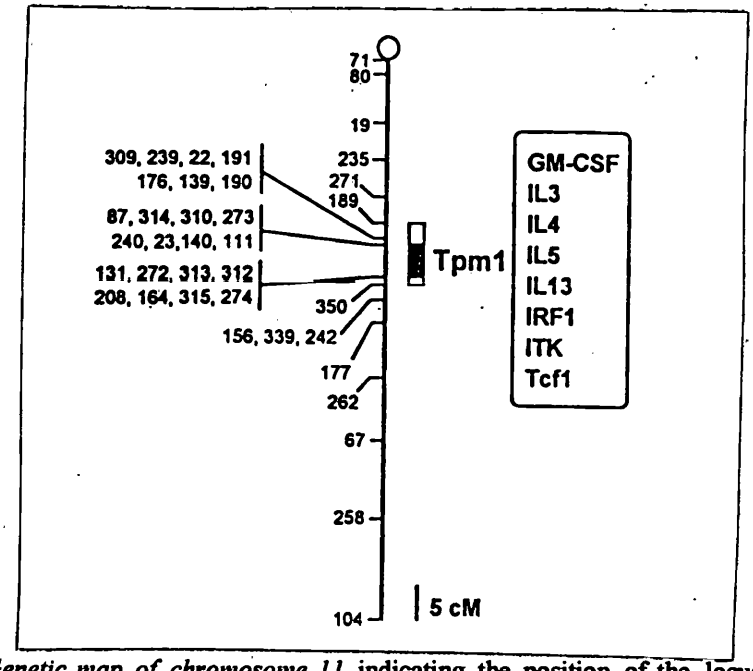

Figure 2. *Genetic map of chromosome 11* indicating the position of the locus that controls maintenance of IL-12 responsiveness (from Gorham et al. 1996 PNAS).
GM-CSF granulocyte-monocyte colony-stimulating factor; IRF-1 interferon regulatory factor-1; ITK, IL-2 inducible T-cell kinase; Tcf1, T-cell factor-1

This chromosomal region is syntenic with a locus on human chromosome previously 5q31.1 shown to be associated with elevated serum IgE levels, suggesting that the regulation of Th1/Th2 differentiation in mouse and of atopy development in humans may be controlled by similar genetic mechanisms (Marsh et al., 1994). Furthermore, multiple allelic forms of the IL-4 promoter were described that exhibit different transcriptional activities in IL-4 positive cells (Song et al., 1996).

1.3) Cytokines

1.3.1) Cytokine induced Th1 development

Cytokines play a crucial role in T helper differentiation under most experimental conditions (Abbas et al., 1996; O'Garra, 1998; Seder and Paul, 1994) (Fig. 3).
IL-12 is a dominant factor in directing the development of Th1 cells (Manetti et al., 1993; Trinchieri, 1995). Consistent with the importance of IL-12 for induction of Th1 differentiation is the finding that IL-12 has striking therapeutic effects in a

number of mouse models of infectious diseases. For example, responsiveness to IL-12 is impaired in BALB/c mice, resulting in a nonhealing Th2 response to *Leishmania major* infection. Administration of IL-12 at the time of infection gives rise to a Th1 protective response (Heinzel et al., 1993; Locksley and Scott, 1991). Furthermore, both IL-12 p40$^{-/-}$ mice and IL-12Rβ1 $^{-/-}$ mice are defective in IFN-γ production and almost completely lack the ability to generate a Th1 response (Magram et al., 1996; Wu et al., 1997).

IL-12 is an heterodimeric cytokine composed of two disulfide-linked subunits, p35 and p40. IL-12 is produced by macrophages upon their encounter with microbial products and intracellular bacteria such as *Listeria monocytogenes* and protozoa such as *Toxoplasma* (Hsieh et al., 1993; Trinchieri, 1995). Recent studies have also shown that Dendritic cells (DC) can produce high amounts of IL-12 upon ligation of CD40 by the CD40 ligand (Cella et al., 1996). Both IL-4 and IL-10 inhibit IL-12 production by APC, thus inhibiting Th1 development (Hsieh et al., 1993).

Interestingly, down regulation of IL-12 production by monocytes has been described as a mechanism developed by measles virus to evade destruction by the immune system (Karp et al., 1996).

In addition to IL-12, interferons act as cofactors in Th1 cell development. Type I interferons are induced primarily as a result of viral infection of cells. IFN-α is largely synthesised by leukocytes, while IFN-β is produced by fibroblasts. IFN-γ is induced by immune and inflammatory stimuli and is synthesised exclusively by T and NK cells (Wu et al., 1997). In mice, it has been shown that neutralization of endogenous IFN-γ in cultures of *naive* $CD4^+$ T cells differentiated in the presence of IL-12 resulted in a significantly reduced IFN-γ production upon restimulation (Bradley et al., 1996; Schmitt et al., 1994). The contribution of IFN-γ to Th1 development can be partially attributed to the ability of this cytokine to induce IL-12 production by macrophages, but it also may have a minor Th1 inducing effect which is independent of IL-12 (see discussion; (Güler et al., 1996)). Macatonia *et al.* investigated the ability of IFNs to guide Th1 development in mice, revealing that

neither IFN-γ nor IFN-α alone can induce Th1 development (Macatonia et al., 1993; Wenner et al., 1996). The role of type I interferons in human Th1 development is still unclear. Allergen specific T cell clones generated in the presence of IFN-α from peripheral blood of atopic patients show a skewing towards the Th0/Th1 phenotype (Parronchi et al., 1996). Furthermore, stimulation of purified resting human T cells in the presence of IFN-α increased the frequency of IFN-γ secreting $CD4^+$ T cells (Brinkmann et al., 1993).

The IFN-γ inducing factor (IGIF, also known as IL-18), a cytokine belonging to the IL-1 family, was also shown to potentiate IL-12 induced Th1 development, despite being unable to drive Th1 development on its own (Robinson et al., 1997). IGIF promotes proliferation as well as IFN-γ production from committed Th1 and NK cells in both humans and mice (Okamura et al., 1995; Robinson et al., 1997; Ushio et al., 1996).

IL-1α has also been described to synergise with IL-12 in Th1 development, but in contrast to IGIF, drives proliferation of Th2 but not Th1 cells (Robinson et al., 1997).

Both IGIF and IL-1α signal through the IRAK pathway, which leads to the activation of NF-kB (Robinson et al., 1997). A molecular mechanism that explains the differential responsiveness to these two cytokines in Th1 and Th2 cells has been recently identified in the differential expression of the IGIF receptor in Th1 cells and in the presence the IL-1R in Th2 cells (O'Garra, 1998). Thus, IGIF and IL-1α, by signaling through NF-kB, may differentially amplify Th1 and Th2 effector responses, respectively.

1.3.2) Cytokine induced Th2 development

IL-4 promotes Th2 development (Le Gros et al., 1990; Seder and Paul, 1994; Swain et al., 1990)(Fig. 3). Knockout of the IL-4 gene results in deficient Th2 responses (Kopf et al., 1993; Kühn et al., 1991).Since it was initially believed that only fully differentiated Th2 cells produce IL-4, it was not clear which cells would produce

IL-4 in order to induce Th2 development. $NK1.1^+$ cells are a small population that recognise antigens presented in association with the non polymorphic β2 microglobulin-associated molecule CD1 (Yoshimoto et al., 1995). These cells produce large amounts of IL-4 rapidly upon activation via anti-CD-3. However a number of studies on β2M-deficient mice failed to support the role of these cells in the development of a Th2 response to a variety of parasitic microbes (Bendelac et al., 1997). Mast cells, basophils, eosinophils and LAK-specific T cell were also indicated as possible sources of early IL-4 (Julia et al., 1996; Paul and Seder, 1994). A recent report suggests that IL-6, a component of inflammatory and acute-phase immune responses, is able to induce IL-4 production from *naive* T cells themselves (Rincon et al., 1997). However, the findings that IL-6 KO mice are capable of developing Th2 responses (Romani et al., 1996) contradict the theory that IL-6 is a good candidate for the Th2 pathway initiator.

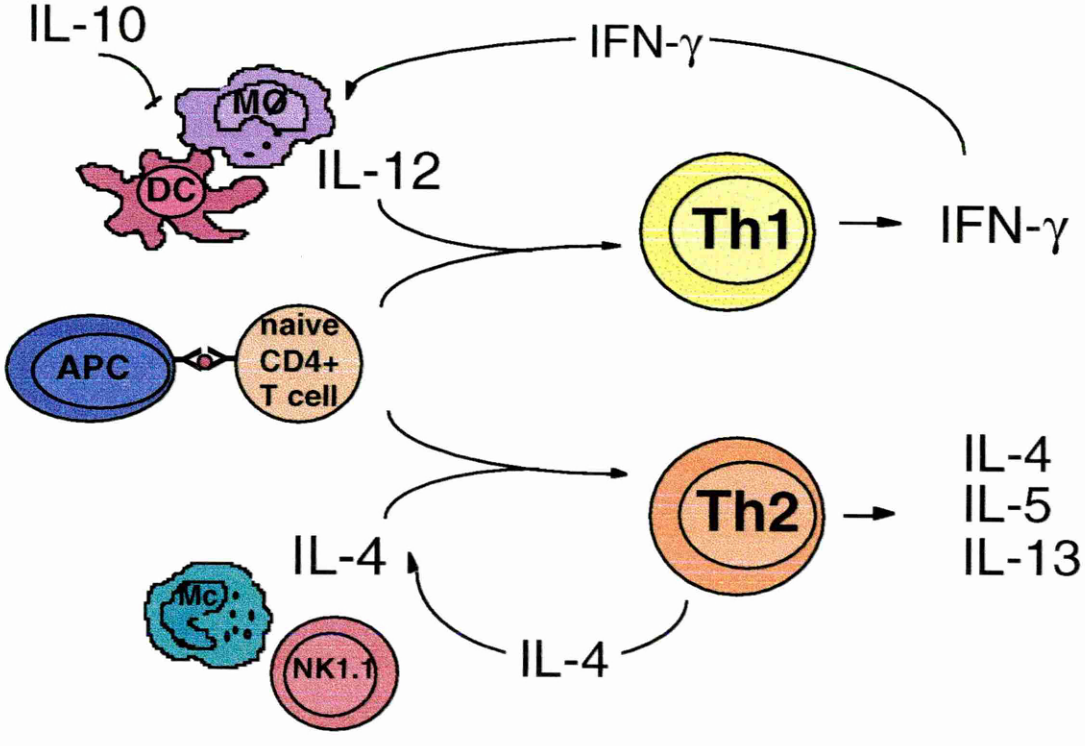

Figure 3. *Cytokine induced T helper development.* MO= macrophage; DC=dendritic cell; APC=antigen presenting cell; Mc=Mast cell

Thus, the T cells should be still considered the original source of IL-4, beginning from the observation that T cells produce small amounts of IL-4 after their initial activation (Schmitz et al., 1994). Increasing lymphocytic activation would lead to the accumulation of IL-4 at the response site, thus reaching a threshold of IL-4 concentration that initiates Th2 differentiation (Abbas et al., 1996). This would be consistent with the observation that Th2 responses become increasingly pronounced with repeated T cell stimulation (De Wit et al., 1993).

Taken together, these findings suggest a model of Th development in which, in the presence of stimuli that trigger macrophages and DC to produce IL-12, such as intracellular bacteria or viruses, a Th1 differentiation pathway is initiated. Conversely, antigens that are persistent or are present at high concentrations, such as helminthic parasites, and do not elicit high amounts of IL-12, would tend to induce Th2 development.

Once T cells begin to differentiate along a particular pathway, the cytokines they produce amplify their growth and development, suppressing the reciprocal pathway. This implies that a selective gene activation pathway takes place along Th differentiation, which is yet almost unknown.

1.4) Transcriptional events controlling T helper cell differentiation

Very little is known about the molecular mechanisms by which expression of the IFN-γ and IL-4 genes is restricted to the appropriate Th subset. The first important observation comes from the analysis of cytokine transcriptional profiles characteristic of Th1 and Th2 subsets during *in vitro* differentiation. Lederer and colleagues investigated the kinetics of IL-2, IL-4, and IFN-γ mRNA expression in early developing Th1 and Th2 cells, after stimulating naive T cells in the presence of IL-12 or IL-4, respectively. IFN-γ mRNA expression was induced as early as 6 h after initial stimulation in the presence of IL-12, and IL-12 treated cultures expressed significantly higher levels of IFN-γ transcripts than control or IL-4 treated cultures. In contrast, IL-4 transcripts were not detected until 48 h after

priming in the IL-4 treated cultures. IL-2 transcripts were detectable 6 h after stimulation in both Th1 and Th2 cultures and expression of the IL-2 gene was not significantly affected by the presence of IL-12 or IL-4, thus indicating that IL-2 expression is not restricted to the Th1 subset (Lederer et al., 1996). This and other studies (Bird et al., 1998), demonstrated that the regulation of IFN-γ and IL-4 gene expression are temporally distinct events and that they may be regulated by independent mechanisms. Thus, considerable efforts were made to identify the molecules, and in particular the transcription factors, that are either selectively present or active in developing Th1 or Th2 cells. Among the few transcription factors characterised so far, most of them are involved in the regulation of the IL-4 gene, while even less is known about the elements involved in T helper 1 development.

1.4.1) NF-AT proteins

Previous studies have demonstrated that the nuclear factor of activated T cells (NF-AT) is critically involved in the induction of IL-2 gene expression in response to TCR-ligation (Shaw et al., 1988). NF-AT is a multisubunit transcription factor consisting of a pre-existing cytoplasmic subunit and an inducible nuclear component containing the activator protein (AP)-1 family members Fos and Jun (Jain et al., 1992). It has been shown that the immuno-suppressive drugs cyclosporin A and FK506 block nuclear translocation of the pre-existing cytoplasmic subunit of NF-AT which occurs in response to antigen receptor triggering(Flanagan et al., 1991). Among the NF-AT family members, NF-ATp and NF-ATc are the predominant NF-AT proteins expressed in human and murine T cells (Lyakh et al., 1997; Rooney et al., 1994). Interestingly, all NF-AT family members have a region of sequence homology with the Rel homology domain of the NF-kB transcription factors. The NF-AT Rel homology domain is believed to interact with polypeptides of the basic region-leucinezipper families, such as Fos and Jun (Nolan, 1994). At least five NF-AT binding sites are present in the

minimal IL-4 promoter and cooperative binding of NF-AT and AP-1 family members to specific sites within the proximal IL-4 promoter were described (Rooney et al., 1995; Szabo et al., 1993). These findings, however, cannot explain Th2-specific expression of the IL-4 gene, since 1) a reporter construct containing multimerised IL-4 promoter NF-AT sites is equally transactivated in both Th1 and T2 clones (Rooney et al., 1995). 2) NF-ATp and NF-ATc are equally expressed in Th1 and Th2 cells (Hoey et al., 1995; Rincon, 1997). 3) analysis of NF-ATp$^{-/-}$ mice revealed impaired expression of the genes encoding IL-2, IFN-γ, GM-CSF, IL-13, and TNF-α and, more surprisingly, increased IL-4 and IgE levels (Hodge et al., 1996; Xanthoudakis et al., 1996). These findings also suggested a negative regulatory role of NF-ATp in IL-4 gene transcription, thus identifying in NF-ATc, the better candidates for IL-4 gene activation.

1.4.2) AP-1, *c-maf* and NIP-45

A functional role for AP-1 family members in T helper differentiation is suggested by studies demonstrating that mutation of the AP-1 sites in the IL-4 promoter results in a 50% decrease in promoter activity (Rooney et al., 1995). Several studies described differential recruitment of AP-1 members to the NF-AT sites in Th1 and Th2 cells, but none have been shown to be exclusively expressed or activated in a Th specific manner (Rincon et al., 1997; Rooney et al., 1995; Szabo et al., 1997).

In an attempt to identify factors that interact with the Rel-homology domain of NF-ATp using the yeast two-hybrid system, Ho *et al.* recently isolated the first Th2 specific transcription factor, the proto-oncogene c-*maf* (Ho et al., 1996). c-*maf* encodes a basic region-leucinezipper protein and shares homology with c-*fos* and c-*jun*. Similar to other AP-1 family members, c-*maf* can form homodimers and heterodimers with Fos and Jun. The evidence that c-*maf* is functionally involved in the regulation of the IL-4 gene stems from experiments in which the cDNA encoding c-*maf* was introduced into a murine Th1 clone and a B cell line. Ectopic

expression of c-*maf* induced IL-4 gene expression in Th1 and in B cells. In addition, overexpression of both c-*maf* and NF-ATp revealed a synergistic effect on the transactivation of the IL-4 gene (Ho et al., 1996). c-*maf* represents the first subset specific transcription factor identified to date. An additional factor interacting with the Rel-homology domain of NF-ATp was recently published by the same laboratory. This factor, designated NIP45 (for NF-ATp Interacting Protein), potentiates transactivation by NF-AT family members and synergises with c-*maf* in the induction of IL-4 mRNA expression (Hodge et al., 1996).

Even though these results can explain Th2-specific expression of the IL-4 gene, the observations that c-*maf* is expressed late along Th2 differentiation and that c-*maf* does not regulate Th2 cytokine genes other than IL-4, raise the question whether c-*maf* is involved in the early polarization of *naive* T cells towards the Th2 subset, or whether the Th2-specific expression of c-*maf* is a consequence of Th2-differentiation.

1.4.3) GATA-3

Recently, using the cDNA Representational Difference Analysis (RDA), the transcription factor GATA-3 was identified as differentially expressed in early developing Th2 cells (Zheng and Flavell, 1997). GATA-3 is a zinc finger DNA binding protein, selectively expressed in T cells and fundamental for T cell development (Tin et al., 1996). GATA-3 is specifically downregulated in developing Th1 cells as early as day 2, thus suggesting a role of this factor in T helper differentiation. Furthermore, GATA-3 is thought to be a more general regulator of Th2 type cytokines since 1) long term Th2 clones expressed a high level of GATA-3 (Zheng and Flavell, 1997). and 2) In GATA-3 transgenic mice, Th1 cells continue to express Th1 type cytokines, but they also express IL-4, IL-5, IL-6 and IL-10 (Zhang et al., 1997; Zheng and Flavell, 1997).

GATA binding sites are present in the IL-5 promoter and GATA-3 activity is sufficient to drive IL-5 expression (Zhang et al., 1998). Less clear is the role of

GATA-3 in the IL-4 regulation. Recent observations showed that the IL-4 promoter is weakly transactivated by GATA-3 directly, but regions in the IL-13/IL-4 locus attribute GATA-3 responsiveness to the IL-4 promoter. Retroviral transduction of GATA-3 into developing T cells only partially restores IL-4 production, thus indicating that GATA-3 is not sufficient to fulfill IL-4 enhancement and additional factors are required (Ranganath et al., 1998; Zhang et al., 1998).

The signals that lead to the upregulation of c-*maf* and GATA-3 have yet to be defined. A potential role of Stat6 in this upregulation is suggested by its rapid activation upon IL-4 receptor ligation and by the demonstrated obligatory role of Stat6 in Th2 development (Kaplan et al., 1996; Shimoda et al., 1996; Szabo et al., 1997; Takeda et al., 1996).

1.4.4) Stat factors

The crucial role of IL-4 and IL-12 signaling in the differentiation of Th subsets has been recently analyzed in Stat6 and Stat4 deficient mice. These proteins are thought to mediate functional responses to IL-4 and IL-12, respectively (see below). Although no significant differences in cell numbers or composition of T- and B-lymphocyte subsets were detectable between wild-type and Stat-deficient mice, Stat6-deficient T lymphocytes fail to differentiate into Th2 cells in response to IL-4 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). In contrast, the analysis of $Stat4^{-/-}$ T cells revealed an impaired production of IFN-γ upon antigen receptor triggering,indicative of a defect in Th1 differentiation (Kaplan et al., 1996; Thierfelder et al., 1996).

Although these studies clearly define a requirement for Stat factors in T helper differentiation, the relationship between Stat activation and T helper development is still uncertain. Lederer *et al.* recently reported that activated Stat6 isolated from Th2 cells could bind a region of the IL-4 promoter which contained a putative Stat6 binding site(Lederer et al., 1996). Even though the functional role of STAT6 in the

induction of IL-4 gene expression needs to be investigated in greater detail, the authors suggest that the observed constitutive activation of STAT6 in developing Th2 cells may be induced by the autocrine effect of secreted IL-4. There is, however, no direct evidence that Stat6 transactivates the IL-4 promoter in T cells or that the Stat6 site of the IL-4 promoter is required for promoter activity. Moreover, exogenous IL-4 does not enhance the expression of IL-4 in mature Th2 cells (Szabo et al., 1997).

Transcriptional activation by Stat factors may also explain Th1-specific expression of the IFN-γ gene. Xu et al. (Xu et al., 1996) analyzed the mechanisms of Stat regulation in response to IL-12. Since IL-12 induces IFN-γ production in activated T and NK cells, they searched for natural Stat binding sites near the IFN-γ gene. DNase I footprinting analysis revealed binding of purified recombinant Stat 1, 4, 5, and 6 to multiple sites within the first intron of the IFN-γ gene. Gel shift assays performed with oligonucleotides encompassing two adjacent Stat4 binding sites from the first intron of the IFN-γ gene revealed strong binding of Stat4. Mutation of either site abrogated Stat4 binding, suggesting that Stat4 bind cooperatively to this element. The domain mediating tetramerization was subsequently mapped to the amino-terminal part of Stat4. Yet, the functional relevance of these findings for the regulation of IFN-γ gene expression is not proven.

1.5) Cytokines signal through the Jak/Stat pathway

Unlike other growth factor receptors, cytokine receptors lack intrinsic catalytic activity. Instead they are associated with a structurally unique class of kinases, the Jaks (O'Shea, 1997).

In the presence of the ligand, receptor subunits oligomerise, the associated Jaks autophosphorylate and in turn phosphorylate tyrosines in the cytoplasmic part of the receptor. This leads to the recruitment of Stat proteins to the receptor where they are phosphorylated on specific tyrosine residues. Upon phosphorylation, Stat proteins dimerise and translocate to the nucleus, where they act as

transcriptional activators by binding specific DNA sequences to the promoters of cytokine inducible genes (Fig. 4) (Darnell Jr, 1997; Ihle, 1996; O'Shea, 1997; Shuai et al., 1994).

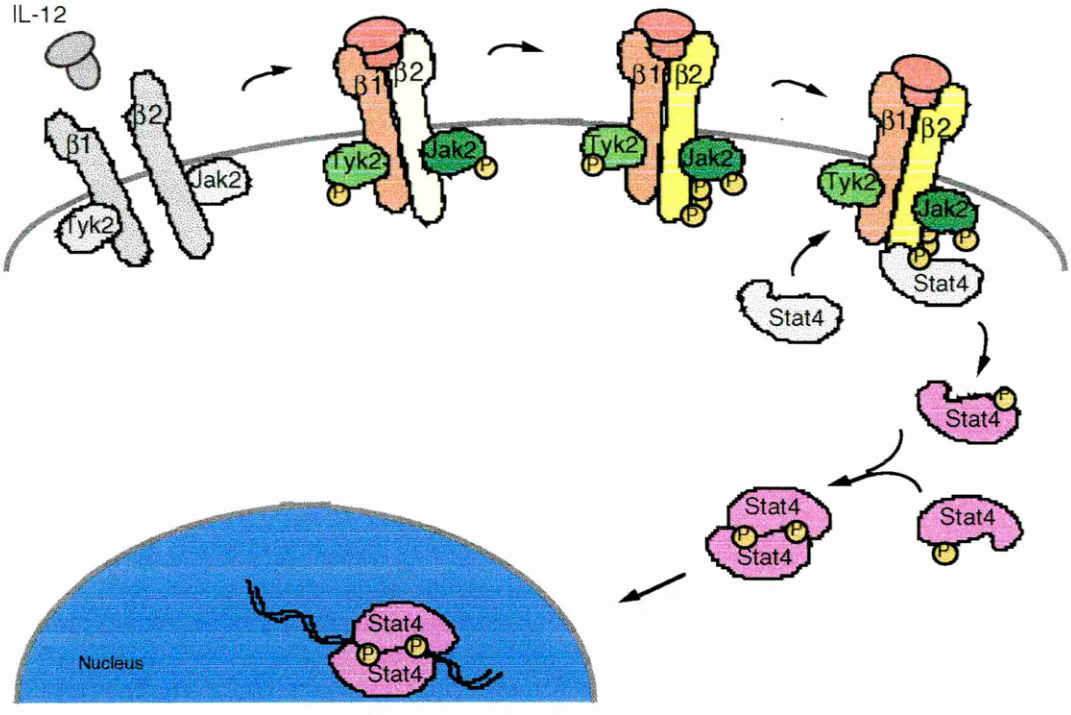

Figure 4. *A model for IL-12Rβ2 signaling pathway.*
The two IL-12R chains β1 and β2 are constitutively associated with the Janus kinases Tyk2 and Jak2, respectively. Binding of IL-12 induces aggregation of the receptor chains and activation of the associated kinases. The activated kinases phosphorylate one or more tyrosine residues present in the cytoplasmic region of the IL-12Rβ2 chain. These phosphotyrosine residues are docking sites for the latent form of Stat proteins. Once bound to the receptor, Stat4 becomes phosphorylated on a single tyrosine residue at the carboxy-terminal region close to the SH2 domain. Reciprocal SH-2 phosphotyrosine interactions between two Stat4 molecules result in the formation of active dimers that translocate to the nucleus and activate gene expression. (Modified from Rogge and Sinigaglia, The immunologist, 1998)

1.5.1) Janus kinases

Jaks are intracellular tyrosine kinases with molecular masses of 120-140 kDa. Four members have been described in mammalian cells: Jak1, Jak2 and Tyk2, which are widely expressed, and Jak3, which is mainly found in cells of haematopoietic origin. The structural organization of Jaks consists of: 1) a kinase domain located at the C-terminus (JH-1 domain); 2) a kinase-like domain (JH2), besides JH-1 and 3) the N-terminal half of the Jaks containing five additional regions with high sequence

similarity between the different Jaks (JH3-JH7) (Schindler and Darnell, 1995) (Fig. 5).

Ligand-induced receptor dimerisation is thought to bring the associated Jaks into close proximity, leading to their activation via inter- or intra-molecular phosphorylation at sites necessary for catalytic activity (Quelle et al., 1994). The finding that Jaks overexpressed in insect or mammalian cells become tyrosine-phosphorylated and display kinase activity even in the absence of an exogenous stimulus indicates that close contact between these enzymes may be sufficient to trigger their activation (Duhe' and Farrar, 1995).

The significance of the kinase-like domain is not clear. It lacks catalytic activity but it can have an influence on the kinase activity, although no clear picture emerges from the literature as to whether this is a positive or a negative one. For example, deletion of the Tyk2 kinase-like domain leads to a protein no longer able to phosphorylate an exogenous substrate (Velazquez et al., 1995). Conversely, mutation in the kinase-like domain of Jak2 resulted in a molecule with increased catalytic activity, suggesting a negative regulatory function of this region (Luo et al., 1997). The N-terminal half of the Jaks (regions JH7-JH3, Fig. 5) is involved in receptor association involving different multiple and discontinuous regions. For example, specific binding of Jak2 to the IFNγR2 is determined by its JH7 and JH6 domains at the very N-terminus (Kohlhuber et al., 1997), while the binding of Tyk2 to IFNαR1 requires the JH7-JH3 domains, and within this region the JH3 and JH6 domains appear to be of particular importance (Yan et al., 1998).

Receptor association of Jaks is thought to be mediated by the membrane-proximal box1/box2 regions that are conserved among many cytokine receptors (Marukami et al., 1991). Box1 is a proline-rich motif of eight amino acid residues essential for Jak association, whereas box2, a cluster of hydrophobic amino acid residues followed by positively charged amino acids, is necessary for Jak association only with some receptors. Also, the region between the two boxes is variably important, depending on the receptor investigated. Some receptors, however, e.g. the IFNRs,

do not fulfill the classical box1/box2 criteria described above, but are still able to bind Jaks (Heinrich et al., 1998).

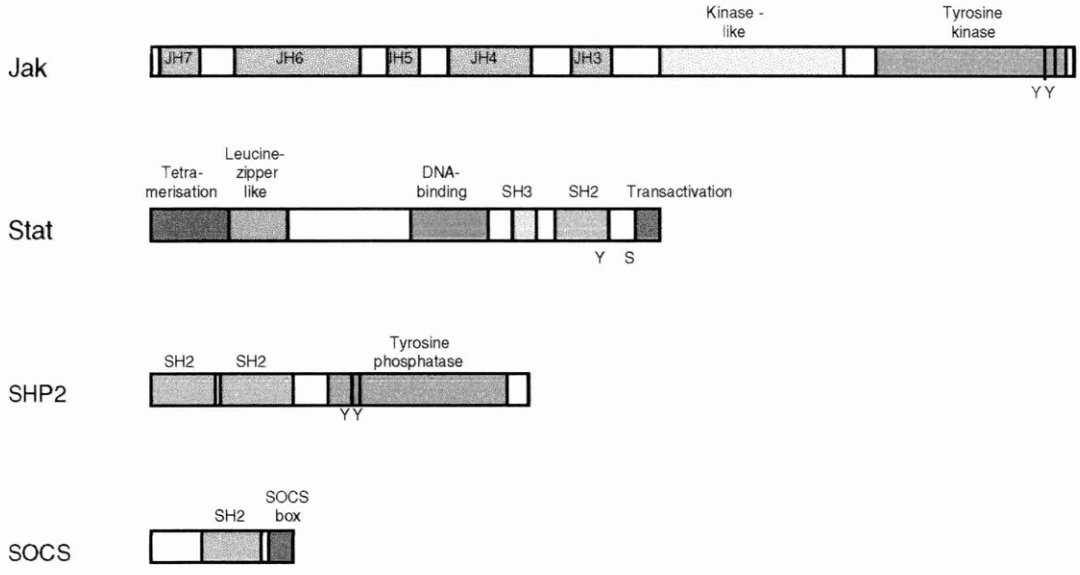

Figure 5. *Structural organisation of Jak-Stat factors, Tyrosine phosphatase SHP2 and SOCS.* Important tyrosine and serine residues that can be phosphorylated are indicated with black bars SOCS=suppressor of cytokine signaling; SHP2= SH2 domain containing tyrosine phosphatase. (Modified from Heinrich *et al*, Biochem. J., 1998).

1.5.2) The Stat family of transcription factors

Seven mammalian Stat genes have been identified so far: Stat1, 2, 3, 4, 5a, 5b and 6. Homologous proteins were also found in Drosophila (D- Stat/marelle) (Hou et al., 1996; Yan et al., 1996) and recently also in Dictyostelium (Kawata et al., 1997). The mammalian Stats localize in three chromosomal clusters, suggesting that this family of proteins has evolved by gene duplication. Since no Stat-like genes have been identified in the genomes of unicellular systems, including yeast, Stats might have evolved with the development of multicellular organisms (Darnell, 1994). Except for Stat2, alternatively spliced forms have been described (Darnell Jr, 1997), however, in the case of Stat4 and Stat6, the corresponding proteins could not be identified.

Stat factors are ubiquitously expressed, with the exception of Stat4, whose expression is more restricted to myeloid cells and testis (Zhong et al., 1994). Stats are activated by cytokine receptors (Schindler and Darnell, 1995), but also by receptor tyrosine kinases (Chen et al., 1997; Park et al., 1996), and G-protein-coupled receptors (Bath et al., 1994).

Like Jaks, Stat proteins have a conserved structural organization that is comprised of: 1) at the C-terminal end an Src homology 3 (SH3)- like domain, an SH2 domain and a transactivation domain, 2) in the middle a DNA-binding domain, 3) at the N-terminus a leucine-zipper-like domain (Fig. 5).

Stat activity is predominantly regulated by post-translational modifications, i.e. tyrosine and serine phosphorylation.

In all Stats, a tyrosine residue near the C-terminus is phosphorylated upon receptor activation. The function of the highly conserved SH2 domain is well established. This domain is responsible for binding of the Stats to the tyrosine-phosphorylated receptor motifs (Chen et al., 1998; Heim et al., 1995) as well as for homo- and hetero-dimerization with other tyrosine- phosphorylated Stat molecules (Shuai et al., 1994).

The phosphorylation of Stat proteins on serine residues is less characterised. Serine phosphorylation is required for full transcriptional activity (Wen et al., 1995). The phosphorylation of serine residues of Stat factors has been associated with signaling components of the MEK/MAPK pathway, thus suggesting that Stat factors can integrate signals from both cytokine and antigen receptors (Ng and Cantrell, 1997; Stancato et al., 1998).

Stat dimerisation is a prerequisite for DNA binding (Chen et al., 1998; Shuai et al., 1994). Stat dimers contact DNA over a 15 bp region (Chen et al., 1998; Horvath et al., 1995). Binding site selection experiments identified a common DNA core recognition consensus sequence defined as $TTCN_3GAA$ (O'Shea, 1997). Stat-binding sites are often arranged as a tandem, suggesting that Stat dimers might form multimers on clustered binding sites. Such multimerization was recently

demonstrated for Stat4, and its N-terminus was found to be essential for this process (Vinkermeier et al., 1996; Xu et al., 1996).

Even though the sequence similarity to functional SH3 domains, the proposed SH3 domain in Stats does not seem to be involved in binding proline-rich sequences, since crucial amino acids are lacking (Zhong et al., 1994).

STATs are activated in the cytoplasm, but they exert their function in the nucleus (Fig. 4), thus, after tyrosine phosphorylation, they have to be transported into the nuclear compartment. Only for Stat1 a nuclear localization sequence (NLS) has been described (Sekimoto et al., 1997), while the other Stat proteins lack this short motif characterized by a cluster of basic amino acids. Thus, nuclear translocation of Stats is achieved either via an atypical NLS or via a shuttle protein that binds to activated Stats and carries an NLS. In this respect the finding that activated Stat5b (Stocklin et al., 1996), as well as Stat3 (Zhang et al., 1997), form a complex with the glucocorticoid receptor (GR) is very interesting, since GR contains two NLSs.

After dimerisation and nuclear translocation Stats bind to specific enhancer sequences and stimulate, and in certain cases possibly repress, transcription of target genes.

The analysis of the promoter region of Stat regulated genes showed that Stat-binding sites are often in close proximity to binding sites for other transcription factors, suggesting a co-operativity of these factors in gene regulation. For Stat3, possible co-operativity with C/EBPβ, nuclear factor (NF)-IL-6 (Schumann et al., 1996) (Stephanou et al., 1998), NF-kB (Brown et al., 1995), activator protein (AP)-1 (Schumann et al., 1996) and GR (Zhang et al., 1997), has been reported.

Stat1 also associates with members of the IRF-1 family (Horvath et al., 1996) and SP1 (Look et al., 1995).

Even though detailed information concerning the Stat-enhancer interaction has become available, it is still not clear how Stat factors and co-operating transcription factors transfer their activation signal on to the basal transcription machinery. In this respect the observation that Stat1 interacts with CREB-binding protein (CBP)

and p300 (Horvai et al., 1997) is very interesting, since CBP and p300 are believed to serve as bridges between transcription factors and RNA polymerase II (Kadonaga, 1998). Notably, these proteins are involved in chromatin silencing/activation through their deacetylase activity. This could represent a link between Stat factors and selective genes activation along T helper development. Thus, although to date some information on gene regulation by Stat factors has been accumulated, the picture emerging is far from clear, and it is expected that, within the next few years, many more details about this crucial process will be worked out.

1.6) IL-12 signal transduction

1.6.1) IL-12 Receptor

IL-12 exerts its biological activity through binding to specific IL-12 receptors that are primarily expressed on activated T and NK cells (Desai et al., 1992).

High affinity (K_d= 5-20 pM, 100-1000 sites per cell) and low affinity (K_d = 2-6 nM, 1000-5000 sites per cell) IL-12 binding sites have been detected on mitogen - activated lymphoblasts (Chizzonite et al., 1992). Two IL-12R subunits have been cloned from human and mouse T cells and designated as IL-12Rβ1 and IL-12Rβ2 (Chua et al., 1994; Chua et al., 1995; Presky et al., 1996). A detailed analysis of ^{125} I-labeled IL-12 binding to BA/F3 cells that had been stably transfected with expression constructs for human IL-12Rβ1 and IL-12Rβ2 revealed that each of the two recombinant subunits binds IL-12 with low affinity, while coexpression of both subunits is necessary to create high affinity IL-12 binding sites. Binding studies performed with the mouse IL-12R constructs revealed that, in contrast to human IL-12Rβ1, mouse IL-12Rβ1 can mediate high- and low- affinity IL-12 binding (Presky et al., 1996). Both IL-12Rβ1 and IL-12Rβ2 subunits are type I transmembrane glycoproteins, with molecular sizes of about 100 kDa (IL-12Rβ1)

and 130 kDa (IL-12Rβ2) and belong to the cytokine receptor class I family. A general scheme is represented in Fig. 6. The extracellular domains are made up of a number of different structural domains. The N-terminus of IL-12Rβ1 consists of the cytokine receptor homology domain. This domain is about 200 amino acids long and contains the N-terminal Cys-Cys pair and the C-terminal WSXWS motif that are characteristic for all cytokine receptors (Stern et al., 1997). The remaining part of the extracellular domains contains three fibronectin type III repeats, each 100 amino acids long (Chua et al., 1994). The extracellular domain of the IL-12Rβ2 is 100 aa longer that the β1, due to the presence of an N-terminal immunoglobulin domain, similarly to gp130 (Presky et al., 1996), to which both IL-12R subunits are highly related. This domain is followed by a 200 aa cytokine domain and three fibronectin type II repeats (Fig. 6). Like gp130 subgroup cytokine receptors, the cytoplamic region of both IL-12R subunits contains the characteristic box 1 motif and a possible box 2 motif. Only the IL-12Rβ2 subunit contains three tyrosine residues. These residues are conserved also in the murine IL-12Rβ2 cytoplasmic region, which also contains additional seven tyrosines. Some of these tyrosines likely play a role in signaling within the cell (see below).

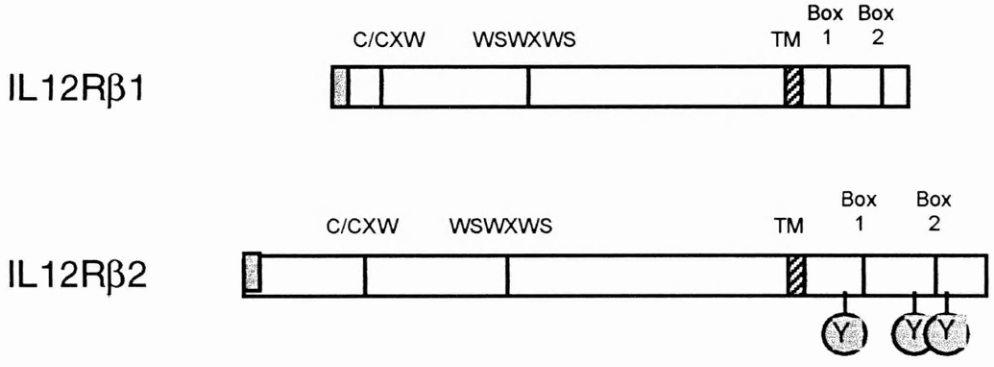

Figure 6. *Schematic illustration of the structural features of IL-12*Rβ*1 and IL-12*Rβ*2.*
The amino acid sequence motifs characteristic of the cytokine receptor superfamily are indicated (C/CXW, WSXWS). TM=transmembrane region.Y= Cytoplasmic tyrosine residues. (Modified from Stern *et al* , Chem. Immunol. 1997)

1.6.2) IL-12 signaling pathway

Ritz and colleagues initially reported the IL-12 induced activation of MAP kinase in mitogen activated human T cells (Pignata et al., 1994), but no further reports confirmed these data. Instead, the IL-12 signaling through the Jak/Stat pathway is well characterised. IL-12 induces tyrosine phosphorylation of Tyk2, Jak2, Stat3 and Stat4 (Fig. 4) (Bacon et al., 1995; Jacobson et al., 1995; Szabo et al., 1995). A differential association between Jaks and IL-12R subunits has been described: Jak2 associates with IL-12Rβ2, while IL-12Rβ1 is most likely associated with Tyk2.

In contrast to Stat3, which is activated by many different cytokines, Stat4 has, thus far, only been found to be activated through IL-12 (Bacon et al., 1995; Jacobson et al., 1995) and IFN-α (Cho et al., 1996). This rather tight link between Stat4 and IL-12 has also been demonstrated in Stat4 -/- mice: these mice are completely unresponsive to IL-12 showing a phenotype that resembles p40 -/- mice (Magram et al., 1996).

It is possible that IL-12 signals through different pathways such as the RAS/Raf one (Pignata et al., 1994). It is not known at the moment whether the different IL-12 activities, such as the induction of IFN-γ production or induction of proliferation, use different signaling pathway.

1.6.3) Differential IL-12 signaling in Th1 and Th2 cells

Using a murine TCR transgenic system, Szabo *et al.* (Szabo et al., 1995) have recently described a novel mechanism controlling the stable commitment to a Th2 lineage. While IL-4 could reverse early Th1 differentiation, IL-12 failed to reverse early Th2 development. In order to examine the reasons for the irreversibility of early Th2 responses, the ability of Th1 and Th2 cells to induce tyrosine phosphorylation of Stats in response to several cytokines was analyzed. While IFN-α and IL-4 activated Stat-containing complexes to a similar extent in both Th1 and Th2 cells, IL-12 selectively activated Jak2, Stat3, and Stat4 in Th1 cells, but

not in differentiated Th2 cells. Thus, reversibility of early Th1 responses results from the maintenance of the IL-4 signaling pathway in the Th1 subset, whereas commitment to the Th2 phenotype results from the rapid loss of IL-12 induced signaling in early developing Th2 cells (Szabo et al., 1995). The question whether IL-12 can reverse established Th2 responses into Th1/Th2 responses was also addressed in human allergen-specific $CD4^+$ T cell clones generated from the peripheral blood of atopic patients. This study showed that human allergen-specific Th cells with a strongly polarized Th2 cytokine profile are not able to phosphorylate Stat4 in response to IL-12, and cannot be induced to produce IFN-γ (Hilkins et al., 1996).

2) Materials and Methods

2.1) Cell culture

T cell lines were grown in RPMI 1640 medium containing 5% Fetal Clone and 100 U/ml IL-2. Jurkat, CEM and 3DO cells were grown in RPMI 1640 medium containing 5% Fetal Clone. COS cells were grown in Dulbecco's Modified Medium containing 5% FC. NK 3.3 cells were cultured in RPMI 1640 medium containing 15% Fetal Calf Serum, 10% Lymphocult and 50 U/ml IL-2. NKL cells were cultured in RPMI 1640 medium containing 15% Fetal Calf Serum and 50 U/ml IL-2.
T2F cells were grown in RPMI1640-CG medium 1:1 containing 100 U/ml IL-2 and 10 % FCS. KIT225/K6 cells were cultured in RPMI 1640 medium containing 5% Fetal Clone and 100 U/ml IL-2.

2.2) T cell lines generation

2.2.1) Generation of PHA blasts from peripheral blood cells

-isolate PBMC from a buffy-coat by Ficoll-Hypaque (Pharmacia Biotech) gradient centrifugation
-suspend PBMC to 1x 10^6/ml in complete medium supplemented with 1µg/ml PHA
-incubate 3 days in a humified incubator 5% CO
-expand them in RPMI 1640 complete medium containing 100 U/ml IL-2 (Hoffmann-La Roche)

2.2.2) Generation of Th1 Th2 lines from cord blood leukocytes

-isolate human neonatal leukocytes from freshly collected, heparinised, neonatal cord blood by Ficoll-Hypaque density gradient centrifugation

-remove $CD8^+$ T cells by positive selection with α-CD8 microbeads and magnetic activate cell sorting according to a protocol supplied by the manufacturer (Miltenyi Biotec)

-resuspend cells at 2 x 10^6/ ml in complete medium and stimulate them in the presence of:

Th1 cultures: 2.5 ng/ml IL-12 (Hoffmann-La Roche) and 200 ng/ml neutralising anti-IL-4 antibodies (Pharmingen)

Th2 cultures: 1 ng/ml IL-4 antibodies (Pharmingen) and 2 μg/ml neutralising anti IL-12 antibodies 17F7 and 20C2 (kindly provided by Maurice Gately)

- on day 3 wash cells 3 times in PBS and expand them in RPMI 1640 complete medium containing 100 U/ml IL-2 (Hoffmann-La Roche)

2.3) Purification of *naive* $CD4^+$ T cells

- purify $CD4^+/CD45RO^-$ T cells from cord blood leukocytes by negative selection using a Pan T cell isolation kit and CD8 microbeads according to a protocol supplied by the manufacturer (Miltenyi Biotec). The purity of the $CD4^+/CD45RO^-$ T cells using this procedure was typically >98% as determined by flow cytometry.

2.4) Single cell analysis of intracellular IFN-γ and IL-4 production

Single cell analysis of IFN-γ and IL-4 production was performed as described previously (PaninaBordignon et al., 1997). Briefly:

- collect T cell lines 7 days after priming, wash once in PBS

- restimulate 10^6 cells with PMA (50 ng/ml) (Sigma, St. Louis, MO) and ionomycin (1 μg/ml) (Sigma) for 2 h at 37 °C in complete medium

-add Brefeldin A (10 μg/ml) (Sigma) to the cultures and incubate for an additional 2 h.

-fix cells with PBS 1% BSA, 2% paraformaldehyde and 0.5% saponin.

-stain cells with anti-huIFN-γ-FITC (Pharmingen, San Diego, CA) and anti-huIL-4-PE (Pharmingen) following the protocol provided by the manufacturer and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

2.5) Immunoprecipitations

-wash 5 x 10^6 cells and perform cytokine induction in 1 ml RPMI-5% FC

- wash cells once in cold PBS

-lyse cell pellet in 250 µl IP-buffer

-incubate 30' on a shaker at 4 °C

-remove insoluble debris by centrifugation (13000 rpm, 4 °C, 30 ')

-add 1µl/ml rabbit polyclonal anti-Stat4 or anti-Stat6 antisera (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and incubate 1 h on a shaker at 4 °C

-add protein A Sepharose and incubate 1 h on a shaker at 4 °C. Cfg 3' 3000 g

-wash four times with cold IP buffer. Centrifuge as above, discart SN

-add 30 µl of loading buffer, boil samples before SDS-page

IP buffer
10 mM TRIS/HCl pH 7.4
150 mM NaCl
1 mM EDTA pH 8.0
1 mM EGTA pH 8.0
1 % NP-40
0.25 % sodium deoxycholate
10 µg/ml Aprotinin, Leupeptin and NaF
1 mM AEBSF and sodium orthovanadate

2.6) Western blot analysis

-transfer gel to nitrocellulose 4h to o.n. in transfer buffer

-incubate o.n. filter in TBST 5% not fat milk

-incubate filter in TBST 5% BSA containing 1µl/ml anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) 1 h on a rotating platform

-wash in TBST 15' on a rotating platform. Repeat three-times

-incubate filter in TBST 5% BSA containing 1µl/ml anti-mouse IgG (H+L) HRP conjugated (Amersham) 1 h on a rotating platform

-wash in TBST 15' on a rotating platform. Repeat three-times

-visualise immunoreactive bands using the ECL Western blotting system (Amersham, Italy), according to the company's protocols.

To control for equal protein loading, blots were stripped and reprobed with anti-Stat4 or anti-Stat6 antisera.

Transfer buffer
380 mM glycine
50 mM Tris base
0.1 % SDS
20% methanol

Tris-buffered saline Tween
20 mM Tris Cl pH 7.4
137 mM sodium chloride
0.1% Tween 20

2.7) Titering Lambda phages

-inoculate 50 ml of LB broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ with *E. Coli* XL1-Blue MRA

-grow overnight with shaking at 30 ^{0}C

-spin the cells down and resuspend them to OD_{600} = 0.5 in 10 mM $MgSO_4$

-make dilutions of the lambda phage in SM buffer

-add 10 ml of the dilutions to 200 ml of host cells for each 100 mm plate

-incubate at 37 ^{0}C for 15 '

-add 3 ml of TOP agar (48^{0}C) and plate on LB plates

-incubate at 37 ^{0}C

LB broth (per liter)
10 g of NaCl
10 g of bactotryptone
5 g of yeast extract

LB plates (per liter)
same as LB broth
add 20 g of bactoagar

TOP agar
same as LB broth
add 0.7% (w/v) agarose

SM buffer (per liter)
5.8 g of NaCl
2 g of $MgSO_4$. H_2O
50 ml of 1M Tris Cl (pH 7.5)
5 ml of 2% (w/v) gelatin

2.8) DNA screening of lambda phages

2.8.1) Primary screening

-plate on large 150 mm plates to 50,000 pfu/plate with 600 ml of OD_{600} = 0.5 host cells/plate and 9 ml of TOP agar/plate. Use 20 plates to screen 1x10^6 pfu

-incubate o.n. at 37 °C (don't leave them grow too much)

-transfer the plates at 4 °C for an hour

-leave NC filters (Amersham) on plates for 1 to 10 '

-mark filters and plates

-dry the filters on the benchtop for at least 10 '

-perform a second lift for each plate

-lay 1-2' on 3MM saturated with

0.2 M NaOH
1.5 M NaCl

-lay 1-2' on 3MM saturated with

0.4 M TrisCl pH 7.6
2x SSC

-lay 1-2' on 3MM saturated with

2x SSC

-dry filters in a oven at 80 °C for 90'-120'

-prehybridise filters 1h at 42 °C with shaking in

5x SSC
50% Formamide
5x Denhardt's
0.5% SDS
400 µg/ml tRNA

-hybridise filters o.n. at 42 °C with shaking in

5x SSC
50% Formamide
5x Denhardt's
0.5% SDS
100 µg/ml tRNA
boiled DNA probe

-wash filters once in 2x SSC 0.1 % SDS 10' 65 °C

-wash filters once in 1x SSC 0.1 % SDS 10' 65 °C

-wash filters twice in 0.1x SSC 0.1 % SDS 10' 65 °C

-dry filters and carry out autoradiography

2.8.2) Secondary screening

-orient the filters, line up the film and mark "dots"(=putative positive clones) with a needle poked though on the corresponding plate

-cut out a square centimeter "window" from the stock plate where the putative clones lines up with the film spot

-put in 500 µl SM buffer and 20 µl chloroform. Vortex

-dilute and titer with host cells on 150 mm plates

-choose single plaque dilutions to make plaque lifts

-perform hybridisation as before

2.8.3) tertiary screening

-pick isolated plaques and perform a third round of screening to obtain phage isolates

-the isolated plaques are resuspended in 500 µl SM buffer and 20 µl chloroform and stored at 4 °C

20x SSC (per liter)
175.3 g of NaCl
88.2 g of sodium citrate
800 ml of water
adjust to pH 7.0 with NaOH

50x Denhardt's
BSA (bovine serum albumin) 1 %(w/v)
Ficoll 1 %(w/v)
polyvinilpyrrolidone 1 %(w/v)

2.9) Lambda phage DNA minipreps

-plate 50,000 pfu/plate on 150 mm plate, grow o.n. 37 °C

-add 10 ml of SM buffer to the plates and resuspend phages incubating on a platform shaker 2h at 4°C

-incubate the supernatant 1h at 37°C with 15 U of DNase I

-centrifuge 1h 30' at 27,000 rpm at 4 °C

-resuspend the pellet in 200 µl of Tris Cl 50 mM pH 7.4

-add 200 µl of phenol pH 8 and incubate with shaking 20'

-extract twice with phenol-$CHCl_3$ and once with $CHCl_3$

-precipitate, dry and resuspend in TE pH 8

2.10) RNase protection assays

-RNA was extracted from T cell lines using Ultraspec total RNA extraction reagent (Biotecx Laboratories Inc., Houston, TX).

-radiolabelled antisense transcripts were synthesized with SP6 polymerase and a commercial kit according to the manufacturer's protocol (Promega, Madison, WI).

-the antisense RNA probes were hybridized to 5 µg total RNA and Ribonuclease protection assays were performed with a commercial kit (Ambion Inc., Austin, TX) according to the company's protocol.

-products were resolved on 6% denaturing polyacrylamide gels and the protected fragments were visualized by autoradiography

-the radioactivity present in the protected fragments was also quantified using a Molecular Imager (Biorad, Richmond, CA). An 18S RNA probe was used as a control for equal RNA loading.

Table I. *Vectors used to derive antisense RNA probes for RNA protection assays*

gene	description	ref
hIL-12Rβ1	553 bp EcoRI/BamHI fragment derived from the IL-12Rβ1 cDNA, subcloned into pGEM-3Z	(Chua et al., 1994)
hIL-12Rβ2	containing a 327 bp DNA fragment encompassing the cytoplasmic region of the human IL-12Rβ2 subunit, cloned in pGEM-3Z	(Presky et al., 1996)
hIRF-1	containing a 400 bp DNA fragment derived from the pUC IRF-1 plasmid (a generous gift of T. Taniguchi), cloned into pBluescript/KS (Stratagene, La Jolla, CA)	(Harada et al., 1994).
hIL-4Rα	hCR-1 Multi-probe template set, (Pharmingen, San Diego, CA)	(Pharmingen, 1996)
hγc	hCR-1 Multi-probe template set, (Pharmingen, San Diego, CA)	(Pharmingen, 1996)
pBET-7	700 bp Taq I fgm of IL-12Rβ2 genomic region, (see fig 1)	this work

2.11) *E. Coli* transformation

2.11.1) Electro-competent cell preparation

-inoculate 1 L.B. broth with 1/100 volume of fresh overnight culture

-grow cells at 37 °C with vigorous shaking to OD600 of 0.5 to 0.8

-harvest cells on ice for 15 ', cfg in a cold rotor at 4000g for 15 '

-resuspend pellets in a total liter of cold water. Cfg as above

-resuspend pellets in 0.5 l of cold water. Cfg as above

-resuspend pellets in 20 ml of cold water. Cfg as above

-resuspend pellets in 3 ml 10% glycerol. Cfg as above

-freeze 40 µl aliquots on dry-ice ethanol. Store at -80 °C

2.11.2) Electro-transformation of *E. Coli*

-chill Biorad 0.1 mm cuvettes on ice

-set Biorad gene pulser to the 25μF capacitor, 1.6 KV and set pulse controller to 200 Ohm.

-thaw 40 μl electro-competent cells and add 1 μl of DNA

-mix and let on ice 1 '

-apply one pulse of the above settings

-immediately add 1 ml SOC medium at room temp

-transfer the cell suspension to 17 x 100 mm polypropylene tubes and incubate 1 h at 37 with moderate shaking

-plate the appropriate aliquots on selective medium

SOC medium
2 % Bacto tryptone
0.5 % Bacto yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgS0_4$
20 mM glucose

2.12) DNA extraction

DNA was extracted using QIAGEN Plasmid Midi, Maxi, Mega and Giga kits, following the manufacturer's instruction

2.12.1) Alkaline lysis minipreps

-pellet 5 ml of o.n. *E. Coli* culture, wash once in TE

-resuspend in 120 μl of the solution:

Tris 25 mM pH 8
EDTA 10 mM
Sucrose 15 %
2 mg/ml lisozyme

-incubate on ice for 10'

-add 240 μl of NaOH 0.2 M SDS 1%. Incubate on ice for 10'

-add 150 µl Sodium Acetate 3 M pH 4.6. Incubate on ice for 20'

-Cfg 15' full speed. Add 1 µl RNase A (1 mg/ml). Incubate 20' at 37 °C

-phenol extract sample twice. Precipitate samples with 2 volumes 100% etOH. Wash with 70% etOH

-dry pellets and resuspend in TE pH 8

When high quality DNA was required (i.e. for sequencing), QIAprep miniprep kit (QIAGEN) was utilised instead.

2.12.2) Dot blot screening

-inoculate single *E. Coli* colonies in 200 µl LB + the appropriate antibiotic selection in a 96 well plate. Grow on at 37 °C.

-transfer 100 µl of the culture to a new 96 well plate, cfg for 5' at 1400 g.

-discart SN and resuspend pellet in 50 µl NaOH 0.4 M

-add 50 µl NH_4COOH 2 M pH 7. Cfg 10' at 1400 g.

-transfer SN to a fresh 96 well plate and add 50 µl 6x SSPE

-prewet filters N+ in 6x SSPE

-set up dot-blot apparatus, wash with 100 µl 6x SSPE

-load 50 µl DNA, wash with 100 µl 6x SSPE.

-dry filters, fix DNA incubating filters in NaOH 0.4 M for 5'

-perform Southern blotting

2.13) Polymerase chain reaction

To isolate and amplify genomic fragments of the IL-12Rβ2 gene PCR reactions were performed utilising PWO DNA polymerase (Boehringer Mannheim) following customer's instructions

Table II. *PCR oligonucleotide primers*

The primers:

forward Nhe I 1223	5'-ATC GCT AGC AGT TAA GGT TGA ACA G-3'
backward Xho I 1699	5'-TAC GGT ACC TAG AGC ATG ATA ATT TC-3'

were used to isolate a 541 nt fragment from pHOP-3 (1223-1699) that contains GAS 3 and GAS 2.

The primers:

forward Acc 65 I 2281	5'-TAC GGT ACC TAG AGC ATG ATA ATT TC-3'
backward Bgl II 2769	5'-ACA AGA TCT CAA ACT CCT GAC CTC-3'

were used to isolate a 488 nt fragment from pHOP-3 (2281-2769) that contains GAS 1 and GAS 1.5.

2.14) Southern blotting

2.14.1) Alkali blotting of DNA

-electrophorese samples in agarose gel, stain with ethidium bromide and photograph with a ruler aid along the gel

-rinse the gel in distilled water and incubate in 0.25 HCl 30' shaking slowly on a platform shaker

-rinse the gel in distilled water and incubate in 0.4 N NaOH 30' shaking slowly onto a platform shaker

-lay the gel on a glass plate, place a positively charged nylon membrane (Hybond-N+, Amersham) and 3 pieces of 3MM Whatman filter paper, all prewetted in 0.4 N NaOH; avoid air bubbles

-stack paper towels on the top, lay a glass plate and place a weight on top

-leave from 2h to overnight

-remove the paper towels and the 3MM Whatman filter paper and recover the membrane, rinse membrane in 2x SSC and allow to air dry

2.14.2) Labeling of DNA

-digest the DNA with the appropriate restriction enzymes in order to obtain a fragment of 300-600 bp, run on a 1% agarose gel and purified using QUIAEX II gel extraction kit

-label DNA using Stratagene's Prime-it II Random Primer Labeling Kit

-purify probe from unincorporated radioactive nucleotides using a nick column (PharmaciaBiotech)

-denature probe 10' at 90 °C just prior adding to the hybridisation solution

2.14.3) Filter hybridisation

-prehybridise filters in 10 ml of hybridisation buffer containing 400 µg/ml yeast tRNA at 65 °C for 4h-overnight

-hybridise filters in 10 ml of hybridisation buffer containing 100 µg/ml yeast tRNA and the denatured DNA probe at 65 °C overnight

-wash filters at room temperature in 2x SSPE 0.1 % SDS

at 65 °C in 1% SSPE, 0.1 % SDS

at 65 °C in 0.1% SSPE 0.1 % SDS

-dry filters and carry out autoradiography

Hybridisation buffer

Na_2HPO_4	0.125 M
EDTA 0.5 M	1 mM
conc H_3PO_4	3.6 ml
0.5 M NaCl	0.25 M
SDS	7 %
PEG	10 %
BSA	1 %

20x SSPE

NaCl	3.6 M
$NaH_2PO_4 \times 2H_2O$	0.2 M
EDTA 0.5 M	0.02 M

2.14.4) Labeling oligonucleotides

Mix:

oligonucleotide(4pmol/µl)	2.5 µl
H2O	18.5 µl
Buffer T4 kinase	3 µl
g ^{32}P-ATP (10 mCi/ml)	5 µl
	30 µl

-incubate 40' at 37 °C.

-purify on a NICK column and use it immediately or store it at -20.

2.14.5) Filter hybridisation

-wash filters 1h at room temperature in 3x SSPE/ 0.1 % SDS

-prehybridise filters at least 1h at 42 °C in

6xSSPE
0.5 % SDS
5x Denhardt
100 µg/ml yeast tRNA

-hybridise overnight at 42 °C in

6xSSPE
1x Denhardt
100 µg/ml yeast tRNA
the probe

-wash filters in

6x SSPE 10' at 50 °C
6x SSPE 20' at 50 °C
6x SSPE 15' at 52 °C

Table III. *Oligonucleotides used for Southern Analysis characterisation of phage clones*

B2 60-38	5'-CAC TTT GTT GAC AGC CAT CAG C-3'
B2 660-649	5'-GAT GGC ACA TAC TTT TAG AGG-3'
B2: 887-866	5'-GGC CAT GGT GAA AAT TGA TTC-3'
13:78-98	5'-GCA ATC AGT GCA AGT CTT CAC-3'
B9-3'	5'-CGA ATA TCT TAG CCA CCT TC-3'

2.15) DNAse I hypersensitive sites mapping

2.15.1) Nuclear extract preparation

-prepare buffer D for dialysis and store it in the cold room, prechill the Dounce homogeniser on ice

-collect cells and wash them twice at 4 °C in cold PBS (1300 x 10')

-resuspend the cells in 5 Packed Cell Volume (PCV) volumes of buffer A

-10' on ice

-cfg 10' at 2300 rpm 4 °C

-resuspend the pellet in 2 PCV of buffer A

-transfer to the cooled Dounce, extract nuclei with 30-40 strokes avoiding bubbles, keep constantly on ice. Check the lysis with Trypan Blue

-transfer to a 14 ml. Falcon tube and centrifuge 10' at 3100 rpm at 4 °C

-remove carefully the SN (that is the cytoplasmic fraction that can be eventually stored at -80)

-gently resuspend the pellet

-add 2 PVC of buffer C

-incubate at 4 °C for 15'-30' minutes

-centrifuge 3100 rpm for 30'

-collect the SN and Dialyse in a Slide-A-Lyzer (MW 10,000, 0.5-3 ml.) (PIERCE)

-dialyse in the cold room 2 h. - o.n.

-collect the extract and cfg 20' at 4 °C

-freeze aliquot of the SN in dry ice/etOH , store at -80

Buffer A

Hepes OH pH 8 0.5 M	10 mM
$MgCl_2$ 1 M	1.5 mM
KCl 2.5 M	10 mM

add just before use: AEBSF 1mM final and DTT 1 mM final

Buffer C

Hepes OH 0.5 M pH 8	20 mM

glycerol	25%
NaCl	0.42 M
$MgCl_2$ 1 M	1.5 M
EDTA 0.5 M	0.2 mM

add just before use: AEBSF 1mM final and DTT 1 mM final

Buffer D

Hepes 0.5 M pH 8	20 mM
glycerol	20 %
KCl 2.5 M	0.1 M
EDTA 0.5 M	0.2 mM

add just before use: AEBSF 1mM final and DTT 1 mM final

2.15.2) DNase I treatment and genomic DNA extraction

-collect 1 x 10^8 cells and wash them twice at 4 °C in cold PBS (1300 x 10')

-resuspend the cells in 5 the Packed Cell Volume (PCV) volumes of buffer A

-10' on ice

-centrifuge 10' at 2300 rpm 4 °C

-resuspend the pellet in 2 PCV of buffer A

-transfer to the cooled Dounce, extract nuclei with 30-40 strokes avoiding bubbles, keep constantly on ice. Check the lysis with Trypan Blue

-transfer to a 14 ml. Falcon tube and centrifuge 10' at 3100 rpm at 4 °C

- remove carefully the SN and gently resuspend the nuclei in 250 µl buffer B

-divide in 5 aliquots of 50 µl each and add 100 µl of DNase I dilutions in buffer B (i.e.: none, 5U, 20 U, 100 U 400 U)

-incubate 2' at RT

-stop immediately the reaction adding 100 ml of lysis buffer of the QUIamp Blood Kit (QIAGEN) and follow up the manufacturer 's protocol for the extraction of genomic DNA

-quantify the genomic DNA and digest 15 µg of each sample with the restriction enzyme of choice

-perform Southern blotting

Buffer A

Hepes OH pH.8 0.5 M	10 mM
$MgCl_2$ 1 M	1.5 mM
KCl 2.5 M	10 mM

buffer B

sucrose	150 mM
KCl 1 M	80 mM
Hepes OH 0.5 M pH 7.4	35 mM
K_2PO_4 1 M pH 7.4	5 mM
$MgCl_2$ 1 M	5 mM
$CaCl_2$ 1 M	2 mM

2.16) Electromobility shift assays

2.16.1) Labeling of double-stranded oligonucleotides

-label 12.5 pmoles of the forward oligonucleotide with T4 polynucleotide kinase (see p.) and purify onto a NICK column. Elute in 400 ml TE pH 7.4

-add 40 µl of 10x annealing buffer (Tris Cl pH 7.4 100mM, EDTA 2.5 mM, NaCl 1 M) and 25 pmoles of the backward oligonucleotide

-incubate 5' at 95 °C

-cool down slowly at room temperature and store at -20

2.16.2) Binding reaction

prepare the following master mix for 30 samples:

60 µl buffer D+
60 µl EMSA buffer
60 µl poly(dI-dC) 1 µg/µl
60 µl BSA 1mg/ml
300 µl H_2O
1 µl unspecific ss oligonucleotide
1 µl DTT 1M
2 µl AEBSF 100 mM
2 µl NaOVa 100 mM

add 50,000 cpm probe/sample

-add 2 µl (= 8 mg total proteins) to 18 µl of reaction mix

-in the case of supershift preincubate the extract 20' RT with 3 µg of concentrated antibody

in the case of competitions with cold ds oligoenucleotides add first the cold oligonucleotide to the reaction mix

-incubate at room temperature 15'

-load on an a 5 % acrylamide native gel

Table IV. *Oligonucleotides used for gel shift assays* Double stranded oligos were used to perform gel shift assays. Only the forward strands are indicated. Nucleotide positions are referred to Fig. 20. Nucleotides that matches to the Stat4 binding site are underlined.

-3984-3966	5'-TT ATG TTC ATT TAA CAA GG-3'*
-3292-3274	5'-GA GTT TCC TCA TTA TAA GT-3'*
-3135-3117 (β2GAS4)	5'-AG TTA TTC ATA GAA TAG TG-3'*
-3026-3006 (β2GAS2)	5'-TTG TAT TTC CTA GAA AAG ACT-3'
-2224-2204 (β2GAS3)	5'-CT GTA TTC TCA TAA TTT GT-3'*
-2158-2139	5'-AT CTG TTC CCT CAA AGA AT-3'*
-1965-1946 (β2GAS1)	5'-TTT TAT TTC CTT GAA AAA CAT-3'
IRFGAS	5'-G CCT GAT TTC CCC GAA ATG ATG-3'
FcγGAS	5'-GTA TTT CCC AGA AAA GGA C-3'
SRF	5'-GGA TGT CCA ATT AGG ACA TCT-3'

*the oligonucleotide used in gel shifts contains a dimer of this site.

D+ buffer

Hepes pH 7.91 M	20 mM
glycerol	20 %
KCl 1M	100 mM
EDTA 0.5 M pH 8	0.5 mM
NP 40	0.25 % (w/v)

EMSA buffer

Tris pH 7.5 1 M	100 mM
NaCl 5 M	500 mM
EDTA pH 8 0.5 M	75 mM
glycerol	43%

2.16.3) Whole cell extracts: TOTEX

-stimulate 2 x 10^7 cells in 10 ml of medium (1h for IL-12, 20' for IFN-γ and IFN-α stimulation)

-spin cells 5' 1400 rpm at 4 °C and discart SN

-add 2 ml cold PBS 1mM AEBSF, 1 mM NaOVa

-spin cells 5' 1400 rpm at 4 °C and discart SN

-spin cells 30" 1400 rpm at 4 °C and discart last SN

-add 50 µl of TOTEX buffer and keep on ice 20'

-spin 10' 4 °C full speed to remove membranes

-transfer SN to a new pre-cooled Eppendorf vial

-add 1 volume Do buffer

-freeze aliquots in dry ice/ethanol and store at -80 °C

2.16.4) Protein assay

follow Bio-rad protein assay reagent instructions, briefly:

-dilute Bio-rad solution 1:5 in H_2O

-distribute 1 ml per cuvette and add 1 µl of protein extract

-as standards pipet 1, 2, 4, 8, 16 µl of BSA (1 mg/ml)

-determine O.D. $_{595}$ nm

TOTEX buffer

Hepes OH 0.5 M pH 7.4	20 mM
NaCl 5 M	350 mM
glycerol	20 %
NP 40	1 %
MgCl2 1 M	1 mM
EDTA 0.5 M pH 8	0.5 mM
EGTA 0.5 M	0.1 mM

Do buffer

Hepes OH 1 M pH 7.9	20 mM
glycerol	20 %
EDTA 0.5 M pH 8	0.2 mM
NP 40	0.25 %

add just before use DTT 2 mM, AEBSF 2 mM, NaOVa 2 mM

2.17) Transfection of mammalian cell lines

2.17.1) Electroporation

-split cells the day before transfection

-resuspend cells in RPMI at 1 x 10^8/ml

-add 28 µg of test DNA + 2 µl CMV β-gal DNA to 400 µl of cells (QIAGEN's plasmid MEGA kit)

- transfer in a 0.4-cm gap Bio-rad cuvette and electroporatate at 960 µF and 300 V using a Gene Pulser (Bio-Rad Laboratories).

-add immediately 3.6 ml of complete medium and distribute in 2 wells of a 24 well plate

- incubate cells for 24 hours at 37 °C in 5% CO_2 in a humified incubator, performing the inductions of choise

(20 h induction for IL-12, 4 h induction for IFN-α, IFN-γ, PMA-ionomicine, α-CD3)

-collect cells and assay cell extracts for luciferase and β-galactosidase activity

2.17.2) Liposome mediated transfection of DNA into tissue culture cells

The following liposome based reagent were tested to transfect lymphoid cells:

Lipofectamine (Life Tecnologies)

Lipofectin (Life Tecnologies)

DMRIE-C (Life Tecnologies)

Dosper (Boehringer Mannheim)

Lipotaxi (Life Tecnologies)

Superfect (QIAGEN)

The reagent of choice for Jurkat and CEM cells was DMRIE-C, with the following optimised conditions:

-wash cells once in serum free medium (Optimem, Life Technologies)

- seed cells in six well plate at a density of 2 x 10^6 per well in 0. 2 ml Optimem

-prepare the following solutions:

Solution A: for each transfection dilute 4 µg DNA in 500 ml Optimem.

Solution B: for each transfection dilute 6 µl DMRIE-C in 500 µl Optimem

-mix solution A and B and incubate at room temperature 15 -30 '

-add dropwise to the cells and incubate 5 h at 37 °C in a 5% CO_2 humified incubator

-add 4 ml of complete medium to each well and incubate overnight

-change medium and incubate for further 24 h, performing the inductions of choise

-collect cells and assay cell extracts for luciferase and β-galactosidase activity

First, in order to test IL-12 induced activation in transient transfectants, an IL-12 responsive cell line was required. Various cell lines were tested for Stat4 phosphorylation, transcription of IL-12Rβ2 gene and how easy to transfect they were:

Table V. *Characterisation of IL-12 signaling and optimisation of transfection efficiency of lymphoid cell lines.* pGL-pro vector and pCMVβgal vectors were cotransfectedusing electroporation or liposomes and transfection efficiencies were determined measuring luciferase and βgal activity *constitutively phosphorylated?

Cellline	Stat4 P*	IL-12Rβ2	Electroporation	Liposomes
Jurkat	+/-	-	+	DMRIE-C lipofectin
CEM	-	-	+	DMRIE-C lipofectin
T2F	-	-	+/-	-
KIT 225 K6	+	+/-	+/-	-
NK 3.3	+/-*	-	-	-
NKL	+/-*	-	-	+/- DMRIE-C
3 DO	+/-	-	+	DMRIE-C

2.18) Promoter activity assays

2.18.1) Luciferase Reporter Gene Assay

The Luciferase Reporter Gene Assay (Boehringer Mannheim) was used, following the company's protocol. Briefly:

-wash cells twice in 2 ml PBS

-resuspend the cell pellet in 250 µl lysis buffer, incubate 15' at room temperature

-centrifuge the lysate in a microcentrifuge at full speed to remove cellular debris

-transfer the SN to a new eppendorf and start immediately the chemiluminescent reaction

-measure light emission of a reaction containing 50 µl of extract plus 100 µl of luciferase assay reagent for 10" using a LB 9507 Lumat luminometer (EG & G Berthold)

-normalise luciferase activities against β-Galactosidase activity.

2.18.2) β-Galactosidase Reporter Gene Assay

β–Galactosidase activity was measured using the β-Gal Reporter Gene Assay (Boehringer Mannheim), according to the company's protocol. Briefly:

-incubate 50 µl of the cell extract obtained to detect luciferase activity with 100 µl of substrate reagent for 15'

-transfer tubes in a luminometer and inject automatically 50 µl of initiation solution. After a delay of 5 sec read light emission for 10 sec

Tale VI. *Oligonucleotides cloned in pGL-pro*

1-DIMluc	5'-GAT TTT CCT TGA AAA ACA TAT GTT TCC TTG AAA AG-3'
2-DIMluc	5'-CGC GTA TTT CCT AGA AAA GAC AGT ATT TCC TAG AAA AG-3'
B2-luc	5'-CGC GTG AGT ATT TCC TAG AAA AGA TCT GTA TTC TCA TAA TTT GTT TTA TTT CCT TGA AAA AG-3'
B2-12-luc	5'-CGC GTG AGT ATT TCC TAG AAA AGA TCT TAT TTC CTT GAA AAA-3'

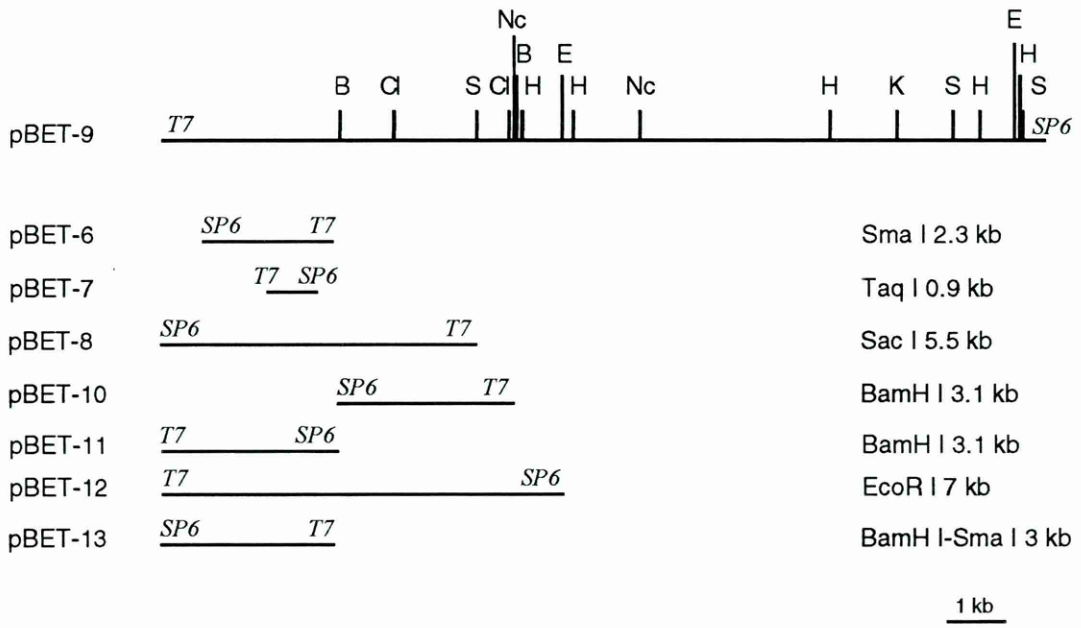

Figure 1. *λFIX4 insert subclones.* λFIX4 Sal I 16.5 kb insert was cloned in pGEM-3Z and restriction mapping analysis performed. This construct was further subcloned and the resulting vectors are indicated. (B=BamH I; Cl=Cla I; E= EcoR I; H=Hind III; K=Kpn I; Nc=Nco I; S=Sal I)

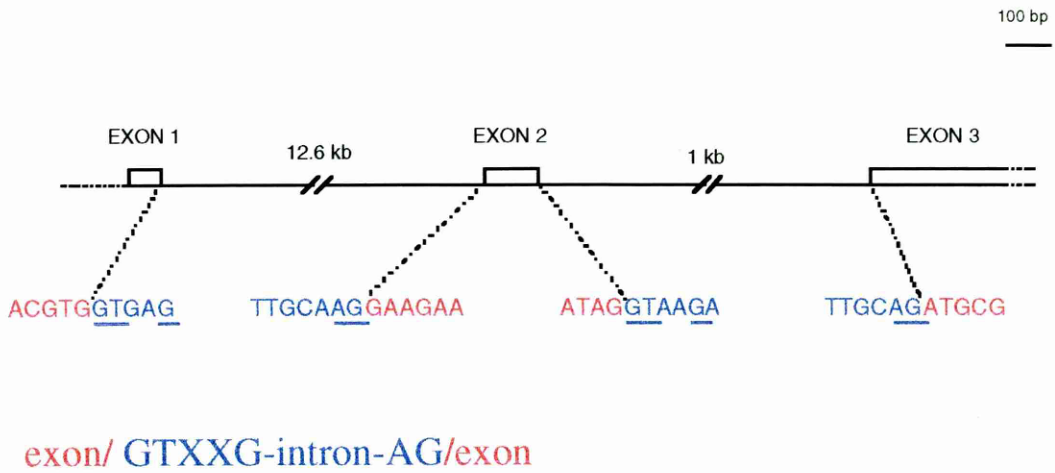

Figure 2. *Intron-exon organisation of the IL-12Rβ2 gene* as determined by subcloning and sequencing. The splice consensus donor and acceptor sites are indicated.

3) Results and Discussion

3.1) Rapid extinction of the IL-12 signaling during Th2 development: characterisation of the molecular mechanisms involved

3.1.1) Analysis of the IL-12 signaling pathway in human Th1 and Th2 cells

To characterize the role of IL-12 and other cytokines in the differentiation of T helper cell subsets, I have generated Th1 and Th2 lines by stimulating human cord blood leukocytes with mitogen in the presence of IL-12 and neutralizing anti-IL-4 monoclonal antibody (mAb) or IL-4 and neutralizing anti-IL-12 mAb, respectively. This protocol allows the establishment of human T cell lines with strongly polarized cytokine production (Fig. 1).

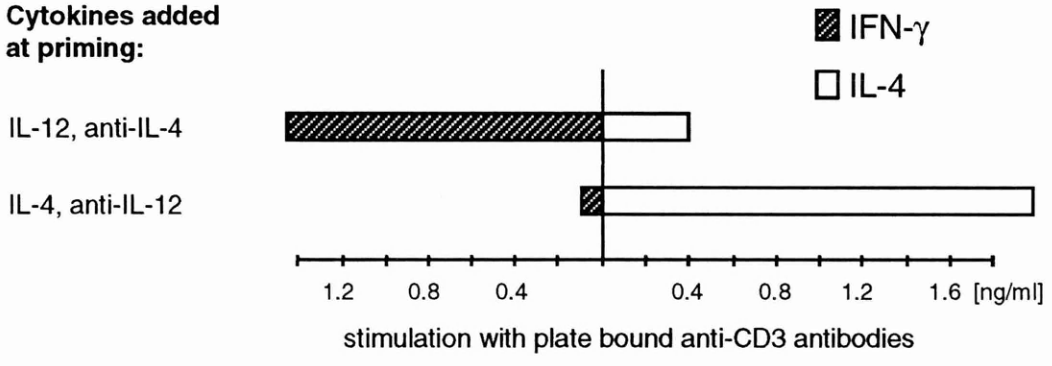

Figure 1. *Cytokine profiles of cord blood derived Th1 and Th2 lines.* IFN-γ and IL-4 production by Th1 and Th2 lines generated from cord blood lymphocytes. Cells were washed on day 10 after priming, 10^5 cells were restimulated in 96-well round-bottom plates for 24h with plate-bound anti-CD3 antibodies (clone TR66 (Lanzavecchia and Scheidegger, 1987)), and IFN-γ and IL-4 in culture supernatants were determined by ELISA assays. The results shown are representative of 5 independent experiments.

Using a murine TCR transgenic system, Szabo *et al.* observed that IL-12 selectively activated the Janus Kinase Jak2 and the signal transducers and activators of transcription Stat3 and Stat4 in Th1, but not in differentiated Th2 cells (Szabo et al., 1995). To ascertain whether also human Th1 and Th2 subsets differ in IL-12

signaling, I have examined Stat4 expression and its tyrosine phosphorylation in response to IL-12 in human Th1 and Th2 lines. As shown in Fig. 2, IL-12 treatment induced tyrosine phosphorylation of Stat4 selectively in Th1 but not in Th2 cells, although both cell types expressed comparable amounts of the Stat4 protein (Fig. 2, lower panel) required for IL-12 signaling (Bacon et al., 1995; Jacobson et al., 1995).

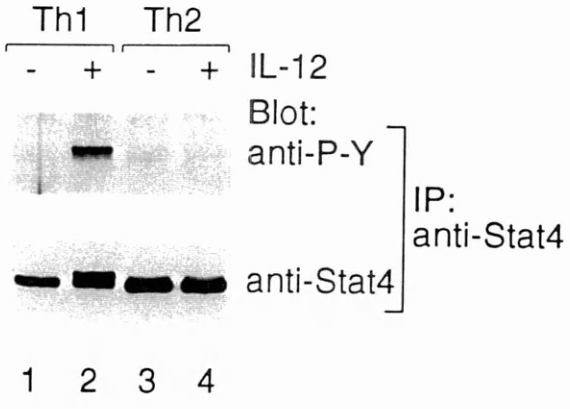

Figure 2. *IL-12 dependent signaling in human Th1 and Th2 cell lines.* IL-12 induces Stat4 phosphorylation in human Th1 but not Th2 cells. Th1 and Th2 lines generated from cord blood lymphocytes were harvested on day 10 after priming. 5 x 10^6 cells were washed and incubated 15 min at 37 °C in medium with or without 2 ng/ml IL-12 followed by the preparation of whole cell extracts and immunoprecipitation with anti-Stat4 antiserum. Precipitated proteins were separated by SDS-PAGE (8%), transferred to nitrocellulose, and probed with anti-phosphotyrosine antibody 4G10 (anti-P-Y, upper panel). As a control for Stat4 expression, blots were stripped and reprobed with anti-Stat4 antibodies (lower panel).

3.1.2) Selective expression of the IL-12Rβ2 chain in Th1 cells

A possible explanation for the observed failure of human and mouse Th2 cells to respond to IL-12 could be the differential expression of IL-12 receptors on Th1 and Th2 cells. To date, two IL-12R subunits, termed β1 and β2, have been identified. Both receptor subunits are required to obtain high affinity binding to IL-12, but

only the IL-12Rβ2 receptor subunit contains tyrosine residues in its cytoplasmic domain required for signaling(Chua et al., 1994; Presky et al., 1996). The mRNA expression levels for the IL-12Rβ1 and β2 subunits were analysed by Ribonuclease protection assays in both Th1 and Th2 lines at different time-points after priming. While IL-12Rβ1 transcripts were expressed in similar amounts in both Th1 and Th2 cells (Fig. 3, upper panel), the transcripts coding for the IL-12Rβ2 subunit were selectively expressed in Th1 but not Th2 lines (Fig. 3). Maximum expression in Th1 cells was seen between day 3 and day 8 and declined thereafter. Th2 cells, in contrast, expressed little IL-12Rβ2 transcripts on day 3, and none were detected after day 8.

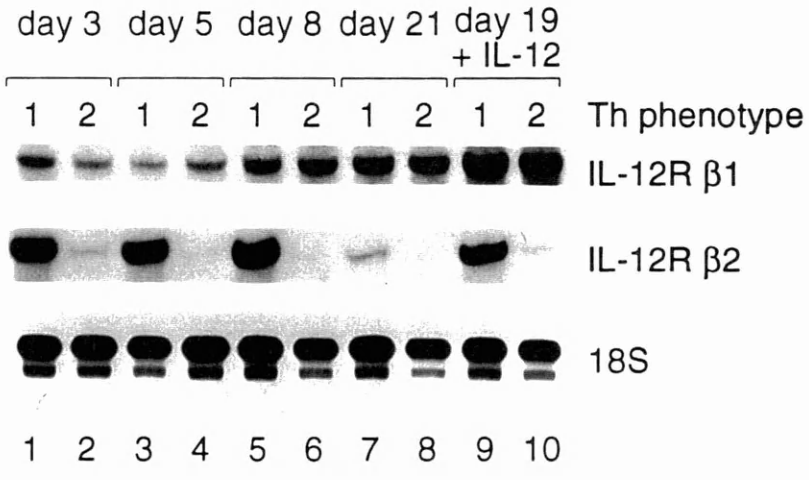

Figure 3. *Transcripts encoding IL-12 receptor subunits in human Th1 and Th2 cells.* IL-12Rβ2 transcripts are selectively expressed in human Th1 cells. Th1 and Th2 lines generated from cord blood lymphocytes were harvested on the indicated time after priming. In lanes 9 and 10 cells were washed on day 19 and incubated for 48h in medium containing 2 ng/ml IL-12 prior to RNA extraction on day 21. Transcripts encoding the IL-12Rβ1 subunit (upper panel), the IL-12Rβ2 subunit (middle panel) and an 18S RNA gene fragment as loading control (lower panel) were quantitated with ribonuclease protection assays (RPAs) as described in Materials and Methods.

A key question is whether cytokine signaling is required for IL-12R induction or whether stimulation via the TCR/CD3 complex is sufficient to induce IL-12R in *naive* T cells. IL-12R transcripts were therefore analyzed in purified CD45 RA^+ T cells before and after stimulation with plate-bound anti-CD3 mAb. IL-12R transcripts were not found in *naive* T cells (Fig. 4, lane 2), but could be detected as early as 24h after T cell activation (Fig. 4, lane 9). Priming of *naive* T cells in the presence of IL-12 resulted in enhanced expression of IL-12Rβ2 transcripts in this population (Fig. 4, lane 13); priming of cells in the presence of IL-4, in contrast, resulted in very low levels of IL-12Rβ2 transcripts (Fig. 4, lane 14). These data indicate that antigen receptor triggering is sufficient to make *naive* T cells express a functional IL-12R, thus inducing IL-12 responsiveness.

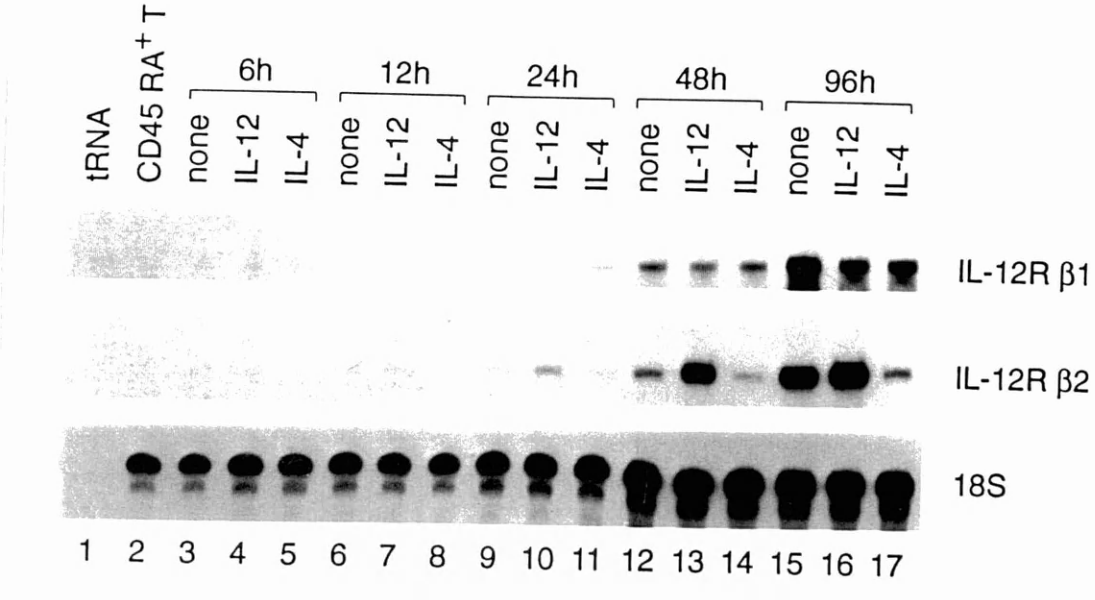

Figure 4. *Antigen receptor triggering induces expression of IL-12Rβ1 and β2 mRNAs in purified CD45RA$^+$ T cells.* CD45RA$^+$ T cells were purified by negative selection from buffy coats as described in Materials and Methods. Purified CD45RA$^+$ T cells (5 x 10^6) were stimulated with plate-bound anti-CD3 mAb with or without the addition of IL-12 (2 ng/ml) or IL-4 (200 U/ml). RNA was extracted from unstimulated CD45RA$^+$ T cells (lane 2) or at the indicated time after CD3-stimulation. Transcripts encoding IL-12R subunits were quantitated in RNase protection assays.

To verify that the expression pattern observed at mRNA level was reflected at the cell surface, flow cytometry analysis of the IL-12Rβ1 and β2 subunits was next performed, as shown in figure 5. Cells were stained with both rat anti-huIL-12Rβ1 mAb (2B10 (Wu et al., 1996)), and rat anti-huIL-12Rβ2 (2B6; (Rogge et al., 1998)). Th1 cell lines, analysed at day 5 after priming (Fig. 5, left panel), both IL-12Rβ1 and β2 subunits were detected. In contrast, only IL-12Rβ1 was found in Th2 cells lines (Fig. 5, right panel). These results demonstrate that the IL-12Rβ2 subunit is selectively expressed in Th1 cells where it is induced upon T cell

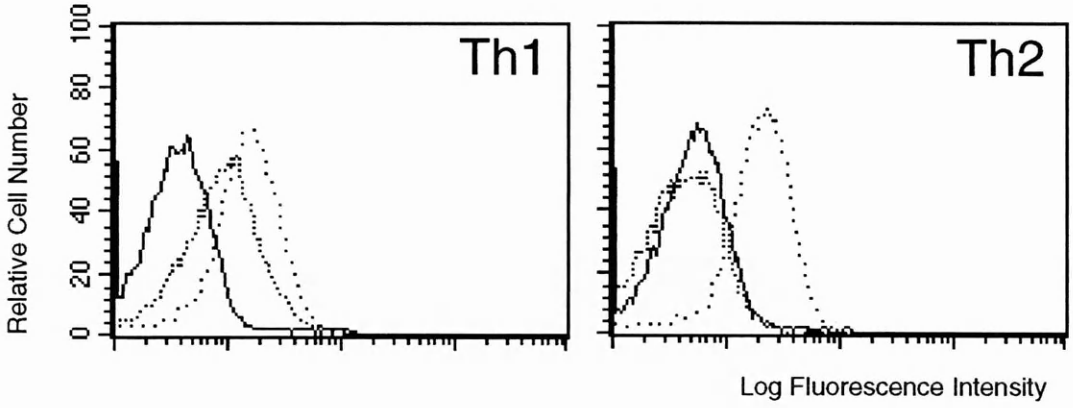

Figure 5. *Cell surface expression of IL-12 receptor subunits on human Th1 and Th2 lines.* CD45RA$^+$ T cells were purified by negative selection from cord blood and stimulated with plate-bound anti-CD3 mAb in the presence of the indicated cytokines and anti-cytokine mAbs. Cells were harvested 5 days after priming and cell surface expression of IL-12Rβ1 and β2 subunits was analyzed with rat-anti huIL-12Rβ1 mAb 2B10 (dotted line), and rat-anti huIL-12Rβ2 mAb 2B6 (stippled line). The solid line represents staining with an isotype-matched control mAb.

activation. Moreover, these data suggest that the lack of IL-12Rβ2 chain expression could be the reason for the inability of IL-12 to activate the Jak2-Stat4 pathway in Th2 cells.

3.1.3) Regulation of IL-12Rβ2 mRNA expression during T helper cell differentiation.

The expression of several cytokine receptors has been shown to be regulated according to positive or negative feedback pathways (Stern et al., 1997). To

determine whether IL-12 directly influences expression of the IL-12Rβ2 transcripts, IL-12 was added to Th1 lines at day nineteen after activation. Culturing the cells in the presence of IL-12 for 48 hours resulted in a 7-fold increase in IL-12Rβ2 transcripts (Fig. 6, lane 2). The IL-12-induced upregulation of the IL-12Rβ2 transcripts was not affected by the presence of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 6, lane 3). These data indicate that IL-12 alone is able to upregulate its own receptor and that this is a direct effect that does not requires *de novo* protein synthesis. Furthermore, Time course experiments revealed that the IL-12 induced upregulation of the IL-12Rβ2 mRNA in primed T cells was transient: maximal induction was seen 24h - 48h after the addition of IL-12 but returned to the level of the control cultures after 96h (data not shown).

Next was examined whether IL-4 also influence IL-12Rβ2 transcription. Neonatal human cells primed in the presence of IL-4 and neutralasing anti-IL-12 mAb completely lost IL-12Rβ2 expression (Fig. 3) and consequently, IL-12-mediated signaling (Fig. 2). The Th cells differentiated in the presence of both IL-4 and IL-12 show a reduction in IL-12Rβ2 expression as compared to Th1 inducing conditions (IL-12 and anti-IL-4)(Fig. 25B).

IL-4 has been shown to block the development of mouse *naive* $CD4^+$ T cells into Th1 effectors (Hsieh et al., 1992; Seder et al., 1992). IL-4 induces the expression of a number of genes of the immune system, such as Fcε receptor II, germline γ1 and IL-1R antagonist (Berton and Linehan, 1995; Kohler and Rieber, 1995; Ohmori et al., 1996), via the activation of the transcription factor Stat6 (Takeda et al., 1996). Inhibitory effects on the regulation of several genes have also been described. For example, Ohmori and colleagues described that IFN-γ-induced activation of IFN regulatory factor-1 (IRF-1) gene is partially suppressed by IL-4 (Ohmori and Hamilton, 1997). This study indicated that the IL-4 suppressive effect is due to competition between IL-4 induced Stat6 and IFN-γ–induced Stat1 for binding to the same GAS site in the IRF-1 promoter. Indeed, binding of DNA of Stat1, but not of Stat6 is sufficient to induce transcription. Similarly, in the regulation of the

E-selectin gene, IL-4 antagonises TNFα- activation of the gene (Bennet et al., 1997). Again, this effect is achieved by competition between Stat6 and NF-kB for binding to the corresponding overlapping sites in the E-selectin promoter.

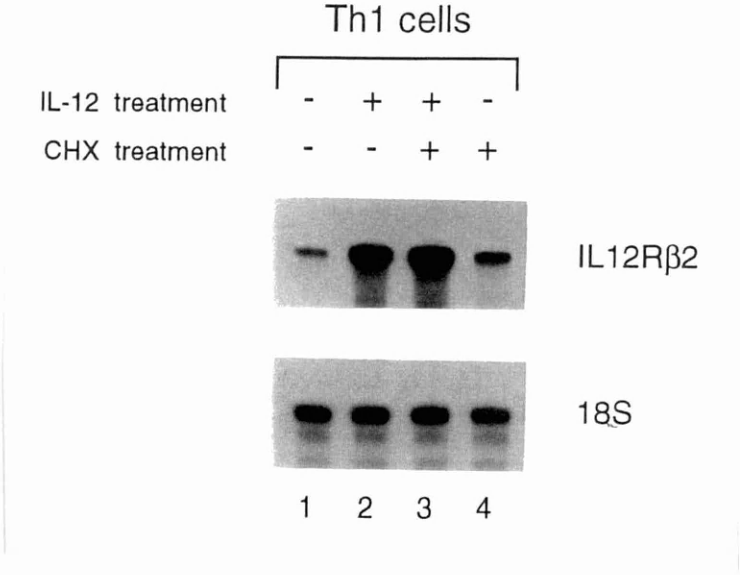

Figure 6. *IL-12 dependent regulation of IL-12Rβ2 transcripts.* Th1 cells generated from cord blood were harvested 2 weeks after stimulation. The cells were treated as indicated with IL-12 (2.5 ng/ml) followed by the extraction of total RNA. To determined whether *de novo* protein synthesis is required for IL-12-induced upregulation of IL-12Rβ2 transcripts, cells were treated with the protein synthesis inhibitor Cycloeximide (CHX, 10 μg/ml) 45 min prior to the addition of IL-12 (2.5 ng/ml) for 16 h. RNA was extracted 16 h after the addition of IL-12. Transcripts encoding IL-12Rβ2 (upper panel) and 18S RNA loading control (lower panel) were analysed by RNase protection assays as described in Materials and Methods.

To test whether IL-4 directly counteracts IL-12-mediated upregulation of IL-12Rβ2 transcripts, established Th1 lines (day 13 after priming) were pretreated for 1 h with IL-4, stimulated with increasing concentrations of IL-12 and after 16h the transcripts for IL-12Rβ2, IL-4Rα and γc were analysed by Ribonuclease protection assays (Fig. 7). IL-4 treatment led to Stat6 phosphorylation (Fig. 8) and to upregulation of IL-4R receptor (Fig. 7, middle panels) (Keegan et al., 1994), thus indicating that functional IL-4 signaling occurs in these cells. Conversely, IL-12-mediated upregulation of IL-12Rβ2 transcripts was not affected by IL-4

pretreatment in all conditions tested (Fig. 7, upper panel). These data indicated that IL-4 does not directly suppress IL-12Rβ2 expression, despite of the fact that these cells are IL-4 responsive.

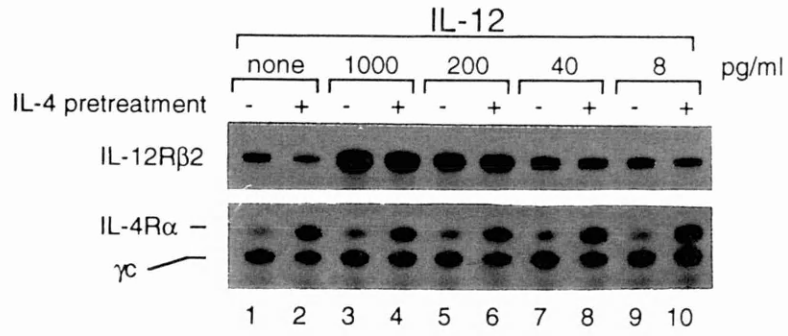

Figure 7. *IL-4 does not directly suppress IL-12Rβ2 m-RNA expression in established T cell lines.* Th1 lines generated from cord blood lymphocytes on day 13 after priming were pretreated (lanes 2,4,6,8 and 10) or not (lanes 1,3,5,7 and 9) with 1 ng/ml IL-4 for 1 h and then stimulated for 16 h with the indicated amounts of IL-12 (lanes 3 to 10). Transcripts encoding the IL-12Rβ2 subunit (upper panel), the IL-4Rα subunit (middle panel) and γc RNA gene fragments (lower panel) were quantitated with ribonuclease protection assays (RPAs) as described in Materials and Methods.

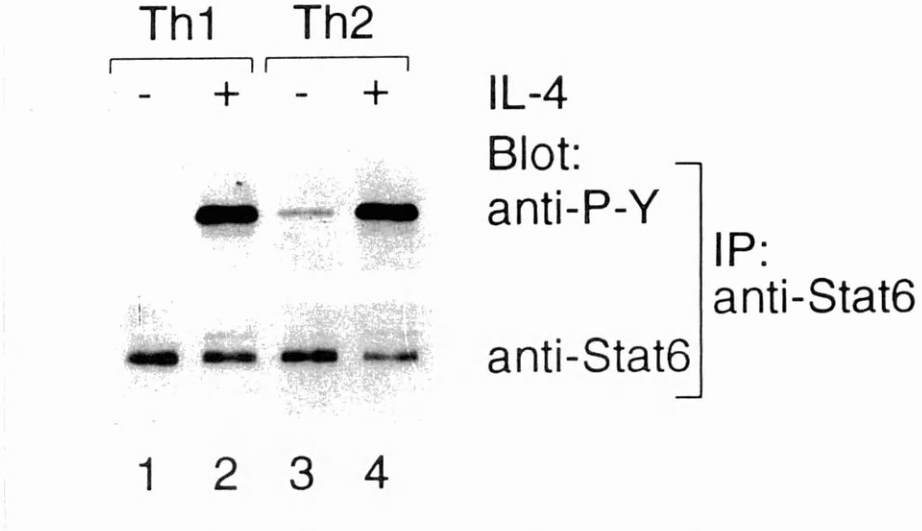

Figure 8. *Stat6 is phosphorylated in Th1 and Th2 cells in response to IL-4.* Th1 and Th2 lines generated from cord blood lymphocytes were harvested on day 13 after priming. 5 x 10^6 cells were washed and incubated 15 min at 37 °C in medium with or without 1 ng/ml IL-4 followed by the preparation of whole cell extracts and immunoprecipitation with anti-Stat6 antiserum. Precipitated proteins were separated by SDS-PAGE (8%), transferred to nitrocellulose, and probed with anti-phosphotyrosine antibody 4G10 (anti-P-Y, upper panel). As a control for Stat6 expression, blots were stripped and reprobed with anti-Stat6 antibodies (lower panel).

3.1.4) Discussion

T helper lymphocytes development is comprised of two spatially and temporally distinct phases of differentiation. Following the first developmental phase, which occurs in the thymus, a second phase, triggered by initial encounter with antigen in the periphery, leads to the development of effector Th subsets producing mutually exclusive pattern of cytokines and playing different roles in the immune response (Mosmann and Coffman, 1989; Paul and Seder, 1994).

Cytokines present at the time of the initial encounter with the antigen, drive the differentiation pathway of the precursor *naive* T cell. In particular, Th1 differentiation is driven by IL-12, while Th2 differentiation is elicited by IL-4 (Abbas et al., 1996; O'Garra, 1998).

To get insights into the molecular mechanisms underlying T helper cell differentiation, I have analysed IL-12 signaling in human Th1 and Th2 cell lines. IL-12 treatment induces tyrosine phosphorylation of Stat4 in human Th1, but not Th2 cells, indicating an early extinction of IL-12 signalingin human Th2 cells. The analysis of the IL-12 signaling components in developing Th cells revealed that the IL-12Rβ2 subunit is selectively expressed in Th1 cells, as observed both at the RNA and the protein level. Thus, the inability of IL-12 to activate the Jak2-Stat4 pathway in Th2 cells is determined by the absence of functional IL-12 receptors on the T cell surface.

Similarly, the selective expression of the IL-12Rβ2 subunit was evidenced in mouse Th1 and Th2 cells differentiated *in vitro* (Szabo et al., 1997). The importance of the IL-12Rβ2 expression for IL-12 signaling was further demonstrated *in vivo,* in the *Leishmania* model: maintenance or loss of the IL-12Rβ2 mRNA by $CD4^+$ T cells strictly correlates with responsiveness or lack of responsiveness in resistant C57BL/6 mice or susceptible BALB/c, respectively (Galbiati et al., 1998; Güler et al., 1996; Himmerlich et al., 1998).

The IL-12Rβ2 chain appears to be the first Th1 subset-specific non-cytokine gene to be isolated to date and it can represent an important marker to identify Th1

cells. Indeed, it has been recently demonstrated in our laboratory that the anti- IL-12Rβ2 mAb recognises lung T cells from patients with sarcoidosis, a disease characterised by Th1 type inflammatory response. In contrast, IL-12Rβ2 was not found on lung T cells from patients with allergic asthma, a desease characterised by type-2 inflammatory response (Rogge et al., 1999).

There is now a growing evidence that cell types other than $CD4^+$ T cells can differentiate in distinct cell subsets that produce type I or type II cytokines. $CD8^+$ Tc1 or Tc2 subsets can be generated *in vitro* (Sad et al., 1995), this polarised phenotype is persistent *in vivo* and $CD8^+$ subsets have been identified in humans as well as in mice in various immunopathological conditions (Cervenka et al., 1998; Seder and Le Gros, 1995). In addition, NK cells have also been recently described to differentiate in distinct cell subsets: NK grown in IL-12 produce IL-10 and IFN-γ, whereas NK grown in IL-4 secrete IL-5 and IL-13 (Peritt et al., 1998). Thus, cell types that were though to be restricted to a type I pattern of cytokines can also produce cytokines of the type II subset, yet their functional significance is still unclear, as both Tc2 and NK2 cells maintain cytotoxic activity. Interestingly, IL-12R modulation in NK2 and Tc2 cells resembles the one observed in $CD4^+$ cells, confirming the key role of IL-12Rβ2 gene in the generation of different cells subsets producing distinct cytokine patterns (Peritt et al., 1998).

The analysis of the transcriptional regulation of the IL-12Rβ2 subunit provided insights in how the expression of a functional IL-12R is obtained.

First, the IL-12R subunits are not expressed in *naive* T cells, but T cell activation is sufficient to induce their transcription. Second, depending on the cytokines present at the time of priming, positive or negative regulatory signals sustain or inhibit IL-12Rβ2 subunit expression thus allowing the development of either a Th1 cell that responds to IL-12 by producing IFN-γ in response to IL-12 or of a IL-12 unresponsive Th2 cell (see Fig. 9).

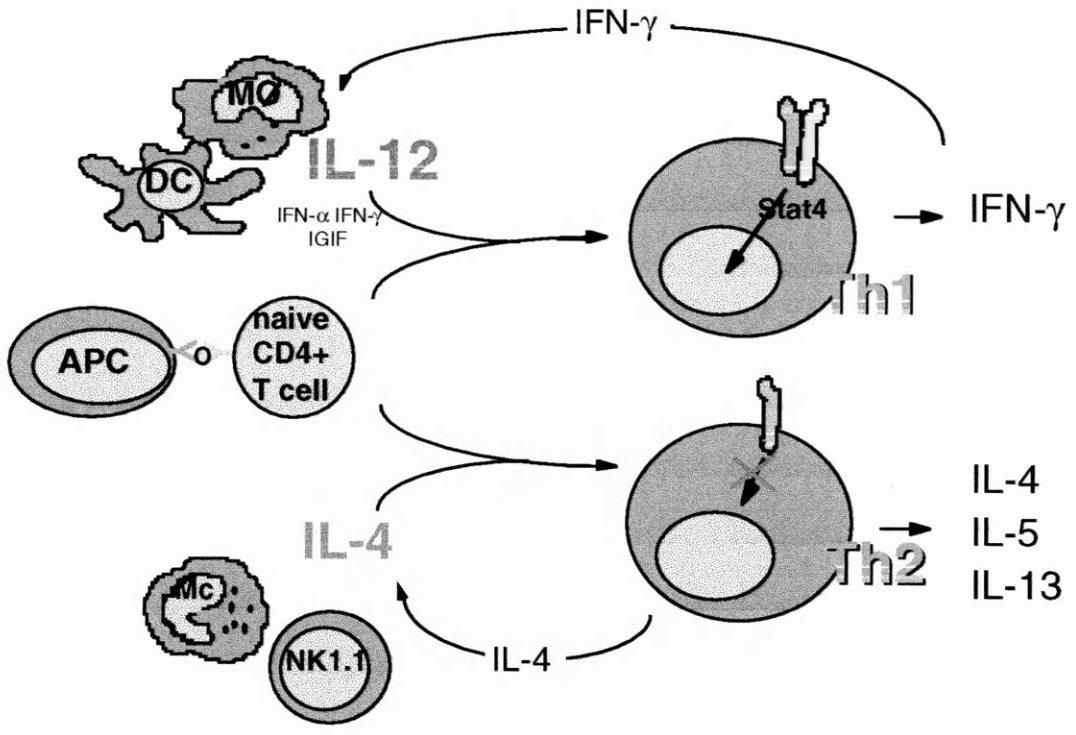

Figure 9. *IL-12Rβ2 subunit expression in cytokine-induced T helper development.* The IL-12R subunits are not expressed in *naive* T cells, but T cell activation is sufficient to induce their transcription. Depending on the cytokines present at the time of priming, positive or negative regulatory signals will sustain or inhibit IL-12Rβ2 subunit expression thus allowing the development of either a Th1 cell that is IL-12 responsive and that produces IFN-γ in response to IL-12 or of a IL-12 unresponsive Th2 cell.

IL-12 is a positive regulatory factor of the IL-12Rβ2 subunit. Not only T cell priming in the presence of IL-12 leads to a strong upregulation of IL-12Rβ2 transcripts, but also IL-12 treatment alone, even when *de novo* protein synthesis is blocked, determines IL-12Rβ2 increased expression. These data indicate that IL-12-induced upregulation of the IL-12Rβ2 gene is a direct effect, that activates preexisting factors in the cell. However the identity of these factors remained unclear (see chapter 2).

T cells differentiated in the presence of IL-4 do not express IL-12Rβ2 transcripts. Th cells differentiated in the presence of both IL-4 and IL-12 show a reduction in IL-12Rβ2 expression if compared to Th1 inducing conditions.

I have been interested in determining whether IL-4 plays a direct role in downregulating IL-12Rβ2 expression. In particular, I have examined the possibility that Stat6, once activated by IL-4, acts as a negative regulatory factor (Bennet et al., 1997; Takeda et al., 1996). The data clearly demonstrated that IL-4 pretreatment of Th1 cells did not affect IL-12-induced upregulation of IL-12Rβ2 transcripts. This is not due to a lack of IL-4 signaling in Th1 cells since these cells: *i)* the IL-4R components are expressed, *ii)* Stat6 is phosphorylated in response to IL-4, *iii)* Stat6 binds DNA in response to IL-4 and more importantly, *iv*) IL-4 upregulates the IL-4Rα subunit. An IL-4 signaling impairment in Th1 cells has been recently reported (Huang and Paul, 1997; Kubo et al., 1997). Huang and colleagues described that repeatedly stimulated mouse Th1 cells, despite the expression of IL-4R subunits at their surface, do not efficiently phosphorylate Stat6 in response to IL-4. Nevertheless, they also describe that, after one-round of stimulation, Th1 cells are still IL-4 responsive, in accordance with our findings in the human system and suggesting that IL-4 unresponsiveness is a consequence, rather than a cause of Th1 development.

Although the mechanism by which IL-4 induces the generation of IL-12 non-responsive Th2 cells remains to be clarified, our data argue against a direct effect of IL-4 in the downregulation of the IL-12Rβ2 gene. More likely, the inhibition of IL-12Rβ2 transcription along Th2 development could be indirectly mediated by IL-4 - induced Th2 specific transcription factors. GATA-3 was shown to be selectively expressed by Th2, but not Th1 cells and to augment Th2 cytokines (Ranganath et al., 1998; Zhang et al., 1998; Zhang et al., 1997). Recently, Ouyang and colleagues (Ouyang et al., 1998) evidenced that GATA-3 acts as a repressor of Th1 development and IFN-γ production. This is not simply a secondary effect of GATA-3 on IL-4 production since it occurs also in IL-4 deficient T cells. In addition, GATA-3 represses IFN-γ production only when continuously expressed during initial T cell development but not when reintroduced into Th1 cells after IL-12-induced development. These findings suggest that GATA-3 may regulate an

early step in Th1 development, rather than directly repressing the IFN-γ promoter. This early step could be the regulation of the IL-12Rβ2, since they also show that GATA-3 expression and IL-12 signaling along Th2 development are mutually antagonistic. The analysis of the IL-12Rβ2 promoter region would provide insights in this regards. The observations that GATA-3 is enhanced by IL-4 addition (Zheng and Flavell, 1997) and that in Stat6 -/- mice GATA-3 expression is strongly reduced (Ouyang et al., 1998), suggest a role of IL-4 in GATA-3 regulation.

3.2) Cloning and characterisation of the IL-12Rβ2 promoter region

3.2.1) Cloning of the human IL-12Rβ2 promoter region.

The data obtained so far indicated that the regulation of the signaling component of the IL-12R complex is the focal point for early maintenance or loss of IL-12 responsiveness in developing Th cells. These findings support a model in which triggering of the antigen receptor on *naive* T cells is sufficient for the initial expression of functional IL-12 receptors on activated T cells. Depending on the cytokines present during the differentiation, T cells will develop into IL-12 responsive Th1 cells or IL-12 non-responsive Th2 cells. So far, two factors that positively influence IL-12Rβ2 expression were identified: TCR triggering and IL-12. However, the role of other cytokines, like interferons, in the development of T helper cells and consequently in the regulation of the IL-12Rβ2 gene has to be clarified. More importantly, it still remains to be elucidated how positive and negative signals are integrated to obtain expression of the IL-12Rβ2 gene and consequent development of IL-12 responsive Th1 cells. In order to provide insights into the molecular mechanisms governing T helper development, I have isolated and characterised the IL-12Rβ2 promoter.

The IL-12Rβ2 promoter region was isolated by screening a human genomic library. The probe used was derived from a cDNA clone containing the 5' untranslated region of the IL-12Rβ2 transcript (the 880 bp Nco I-Xba I fragment of clone B5-10, kindly provided by Ueli Gubler, Roche Nutley, (Presky et al., 1996)). Three positive clones were isolated after three rounds of screening: λFIX-1, λFIX-3, λFIX-4. To characterise the inserts of the phages, phage DNA was extracted and Southern blot analysis performed. For mapping studies, oligonucleotide probes of the 5'-end of B5-10, of the start codon and of a downstream region across the Nco I site were derived from the IL-12Rβ2 cDNA (B2 60-38, B2 660-649, B2: 887-866, respectively; see materials and methods).

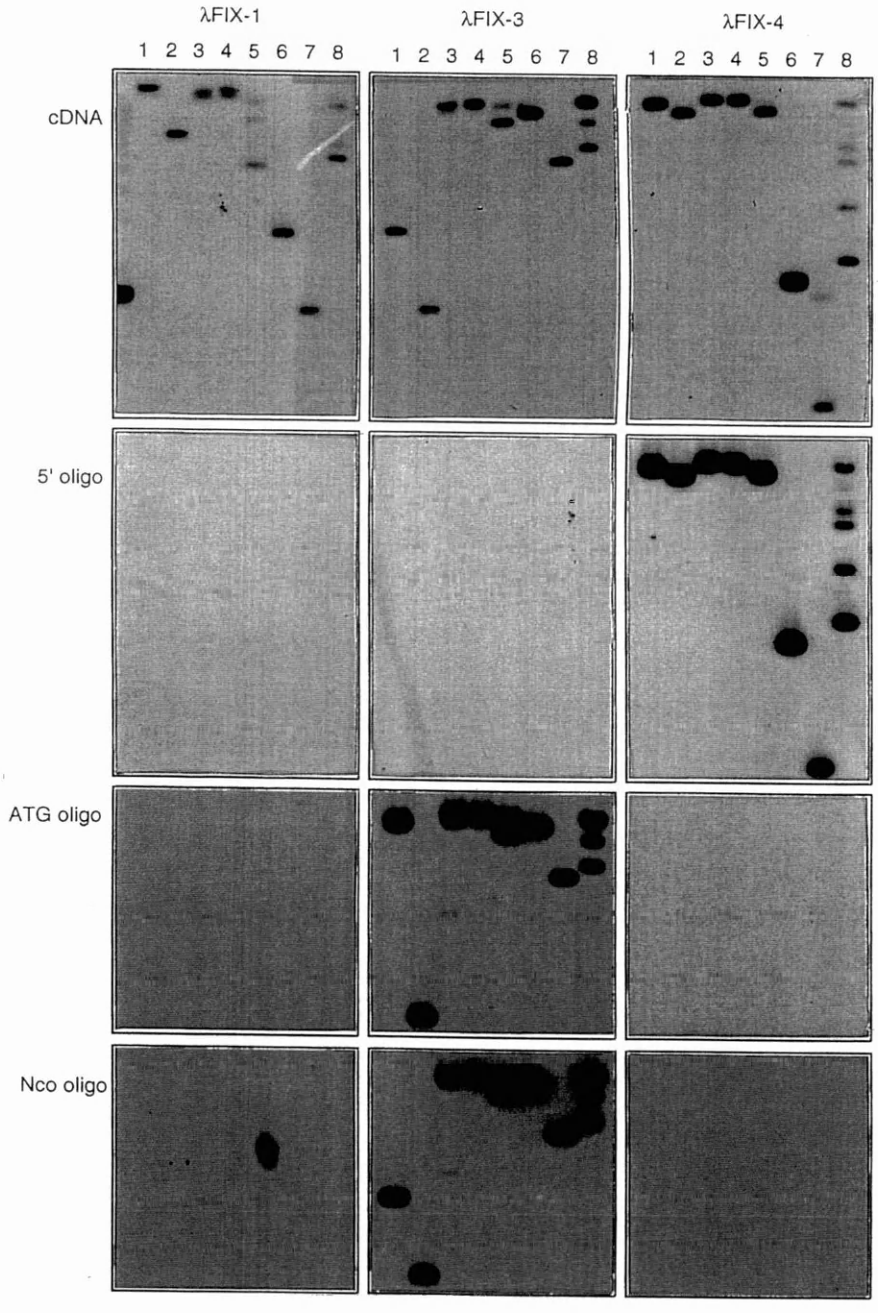

Figure 10. *Southern blot analysis of B5-10 positive phages isolated from a human placenta genomic library.* Phage DNA was extracted from λFIX-1 (left panel) λFIX-3 (middle panel) and λFIX-4 (right panel) and digested overnight with BamHI, Bgl II, EcoRI, Hind III, Not I, Sma I, Taq I Xba I (lines 1 to 8). Southern blot analysis was performed using as probes: **A)** the IL-12Rβ2 cDNA fragment B5-10 (see text), **B)** B2 60-38: 5'-CAC TTT GTT GAC AGC CAT CAG C-3';**C)** B2 660-649: 5'-G AT GGC ACA TAC TTT TAG AGG-3'; **D)**B2: 887-866 5'-GGC CAT GGT GAA AAT TGA TTC-3'.

As shown in figure 10 (left panel) λFIX-1 did not hybridise with any of the oligonucleotides, indicating that it contained downstream sequences of the IL-12Rβ2 gene.

λFIX-3 hybridised with oligonucleotides B2: 660-649 (ATG) and B2: 887-866 (Nco I) but not B2 60-38 (5'), indicating that it contained the start codon, but not the 5' end of the IL-12Rβ2 gene (Fig. 10, middle panel). Conversely λFIX-4 hybridised with the oligonucleotide B2: 60-38 (5'), but not B2: 660-649 (ATG) and B2: 887-866 (Nco I), indicating that it contained the upstream part of the gene (Fig. 10, right panel). The 16.5 kb λFIX-4 insert was excised with Sal I and cloned in pGEM-3Z, generating pBET-9. pBET-9 was further characterised by restriction mapping analysis (Fig. 11 see also materials and methods) and its subclone pBET-13, containing the 3 kb Sal I- Sma I fragment was entirely sequenced (see Fig. 19 and 20).

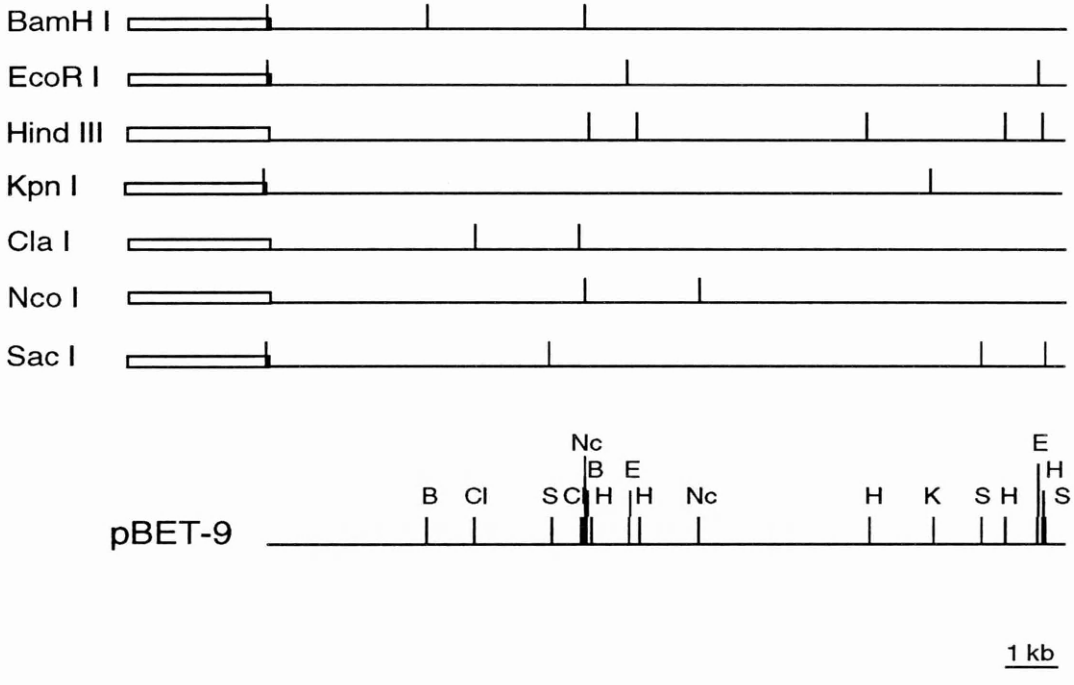

Figure 11. *Restriction mapping analysis of pBET-9.* Restriction map of pBET-9, containing the 9 kb Sal I fragment derived from λFIX-4. The position of the restriction enzyme cut sites was determined performing single and double digestions and Southern blot analysis as described in materials and methods. (B=BamH I; Cl=Cla I; E= EcoR I; H=Hind III; K=Kpn I; Nc=Nco I; S=Sal I).

Since DNase I Hypersensitive site mapping analysis (see below) revealed that regulatory regions were located further upstream the sequence covered by pBET-9, an additional library screening was performed. In order to isolate this region, clones were selected that hybridised to an oligonucleotide probe chosen in the most 5' region of pBET-9 (13:78-98 see materials and methods, Tab. III) but did not hybridise to a 0.5 kb EcoR I-Pvu II DNA fragment (located 3.7 kb downstream the RNA start site). Only λHOP-1 phage fulfilled these requirements and was further characterised. The Sal I-Pvu II 8 kb phage fragment that hybridised with the oligonucleotide probe was subcloned in pBluescript (pHOP-1), then Southern blot analysis and restriction mapping were performed. The restriction pattern of the pHOP-1 partially resembled the one of pBET-9 indicating an overlap between the two inserts. In particular the 3.5 kb Xba I fragment of pHOP-1 was identified through restriction analysis and Southern Blotting as a shorter fragment that contained the region where the hypersensitive site mapped; it was subcloned in pBluescript (pHOP-3) and entirely sequenced. As shown in figure 19 and 20, the sequence analysis of pHOP-3 and pBET-13 allowed the definition of a contig of 5 kb, that contained, as described afterwards, the promoter and also enhancer regions of the IL-12Rβ2 gene.

In order to characterise also the immediate downstream part of the gene and define its intron-exon organisation, I have also investigated whether λFIX-4 and λFIX-3 were contiguous. For this purpose, an oligonucleotide derived from the most 3' region of λFIX-4 (B9-3', see materials and methods) was used to perform Southern blot analysis of λFIX-3. The oligo probe hybridised to λFIX-3, indicating a partial overlap of the two phages. The 1.6 kb Bgl II fragment of λFIX-3 phage, which was hybridising with the oligonucleotide, was subcloned in pBluescriptSK II (pSK-1) and sequenced (see materials and methods, Fig 2).

The contig resulting from the overlap of the phages pHOP-1, λFIX-4 and λFIX-3 allowed the characterisation of a 16.5 kb region of the human genome that contains part of the IL-12Rβ2 gene, as summarised in Fig. 12. The comparison between the

cDNA and the genomic sequences allows the definition of its intron-exon organisation. The hIL-12Rβ2 gene has a first untranslated exon of 547 bp which is separated by a long first intron (12.5 kb) from the second exon (112 bp) that contains the start codon of the gene. The third exon follows after a 1 kb second intron (Fig. 12).

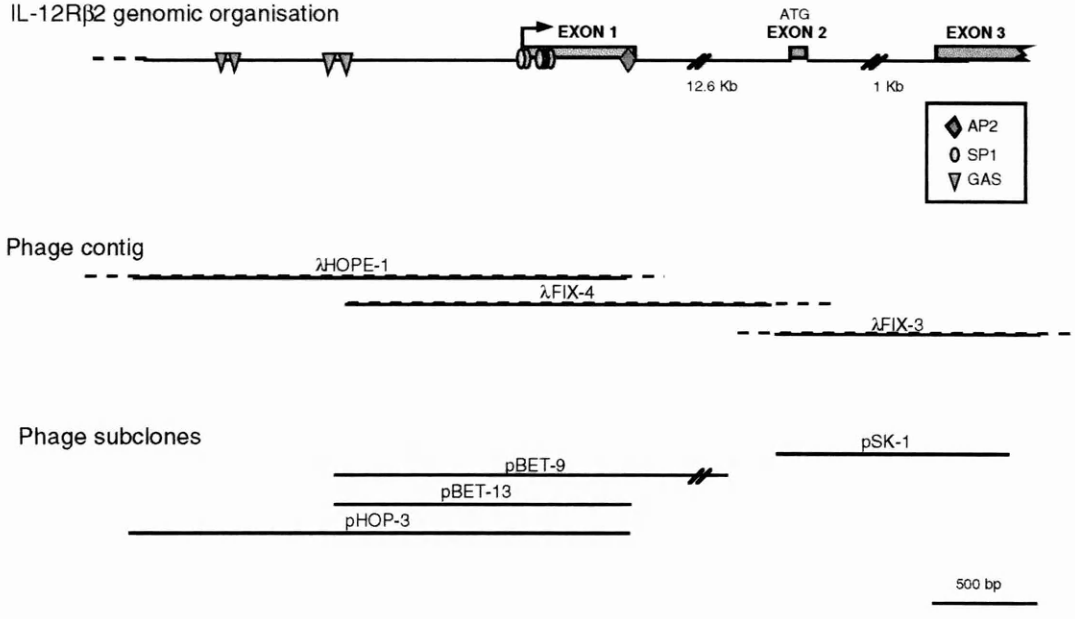

Figure 12. *IL-12Rβ2 genomic organisation.* A 16.5 kb region of the human genome that contains part of the IL-12Rβ2 gene is shown. The characterisation of this region was obtained by restriction mapping and sequencing analysis of the phages pHOP-1, λFIX-4 and λFIX-3 Some of the phage subclones utilised for the analysis of the region are also indicated (see text). The hIL-12Rβ2 gene has a first untranslated exon of 547 bp which is separated by a long first intron (12.5 kb) from the second exon (112 bp) that contains the start codon of the gene. The third exon follows after a 1 kb second intron. The consensus binding sites for transcription factors are also indicated.

In order to verify whether the phage inserts were not containing cloning artifacts, genomic DNA from peripheral blood mononuclear cells (PBMC) was isolated and genomic Southerns were performed (Fig. 13). DNA fragments derived from pBET-9 were used as probes.

The restriction pattern evidenced in the genomic Southern coincided with the one determined from the phage contig, indicating the absence of rearrangements in the phage inserts.

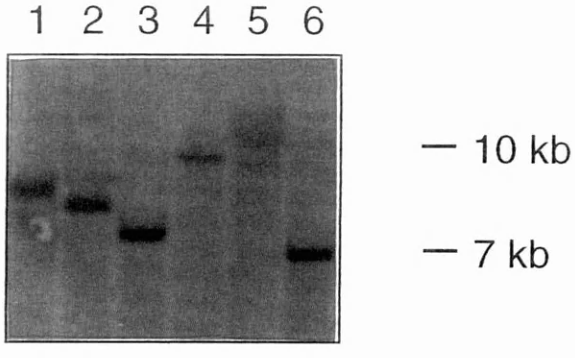

Figure 13. *Genomic Southern analysis of the IL-12Rβ2 gene.* Genomic DNA was extracted from PBMCs as described in materials and methods and digested with EcoR I, BamH I, Sac I, Hind III, Sma I and EcoR V (lanes 1-6). Digested DNA was run in a 0.8% agarose gel and Southern blot analysis performed using the 0.5 kb Pvu II-BamHI probe.

3.2.2) Identification of the RNA start site

Once the genomic region covering the IL-12Rβ2 gene was isolated the first step in the identification of the promoter was the determination of the RNA start site.

To identify the RNA start site of the hIL-12Rβ2 gene, the region in which the 5' of the B5-10 cDNA mapped in the genomic sequence was investigated using Rnase Protection Assays. Radiolabelled sense and antisense RNA probes were derived from a 900 bp Taq-1 fragment encompassing the 5'-end of B5-10 and hybridised to total RNA purified from Th1 or Th2 cells. Transcripts were detected in Th1 (Fig. 14, lane 2), but not in Th2 cells (Fig. 14, lane 3), with the antisense probe. These results indicated that transcription of the IL-12Rβ2 gene starts from multiple sites

in a 30 nt window located at the 5' end of the cDNA clone B5-10 that was originally used to isolate the IL-12Rβ2 gene.

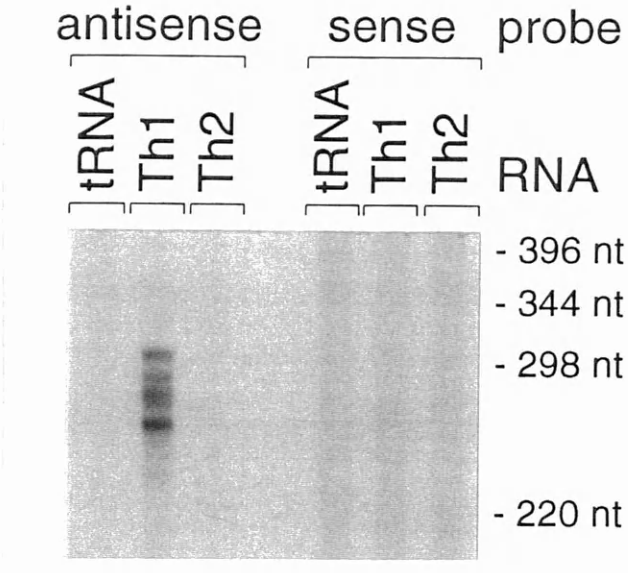

Figure 14. *Identification of the IL-12Rβ2 RNA start site.* To identify the RNA start site of the hIL-12Rβ2 gene, the region in which the 5' of the B5-10 cDNA mapped in the genomic sequence was investigated using Rnase Protection Assays. Radiolabelled sense (left panel) and antisense (right panel) RNA probes were derived from a 900 bp Taq I fragment encompassing the 5'-end of B5-10 and hybridised to total RNA purified from Th1 or Th2 cells.

3.2.3) Mapping of DNase I Hypersensitive sites within the IL-12Rβ2 gene

Analysis of IL-12Rβ2 regulation at RNA and protein level indicates the presence of two major signaling pathways influencing IL-12Rβ2 expression. Triggering of the antigen receptor on *naive* T cells is sufficient for the initial expression of functional IL-12 receptors on activated T cells. The presence of IL-12 at the time of priming maintains IL-12Rβ2 expression and allows development of Th1 cells.

To identify the regulatory regions involved in TCR and IL-12 mediated regulation of the IL-12Rβ2 gene, DNase I Hypersensitive Site (DHS) mapping analysis of the upstream region of the hIL-12Rβ2 gene was performed. This technique is based on the principle that a short digestion of purified nuclei with DNase I leads to the preferential cut of chromatin in the most accessible sites. These sites often correlate with transcription factor binding sites (Weintraub and Groudine, 1976). In this approach a specific genomic probe that hybridises to one end of a restriction fragment was used to probe a Southern blot of Pvu II-digested DNA samples isolated from nuclei that had been digested with increasing concentrations of DNase I.

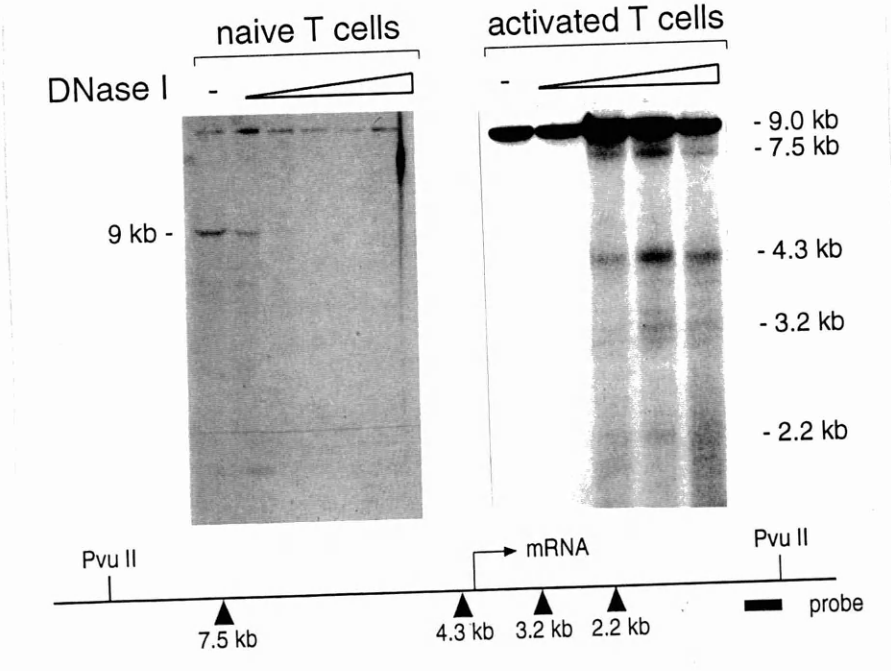

Figure 15. *DNase Hypersensitive sites are present in the IL-12Rβ2 gene in activated PHA blasts, but not naive T cells.* Nuclei were purified from CD4+ *naive* T cells (left panel) or PHA blasts (right panel) and briefly treated with increasing amounts of DNase I (lanes 2-5). Genomic DNA was extracted, 10 μg were digested with Pvu II and Southern blot analysis performed using the 0.5 kb Pvu II-BamH I genomic fragment as a probe.

The resulting autoradiogram shows the genomic fragment of 9 kb flanked by Pvu II sites and specific subfragments generated by DNAse I digest. The position of the DNAse I hypersensitive sites with respect to the Pvu II restriction sites in the IL-12Rβ2 gene can be defined by the length of the respective subfragment. Using this approach I mapped hypersensitive sites encompassing a region that extended 5 kb upstream and 4 kb downstream from the RNA start site (see Fig. 16).

First, I have analysed DNase I hypersensitive (DH) sites in *naive* $CD4^+$ T cells. Only the parental 9 kb Pvu II fragment was evidenced, indicating the absence of DH sites in this region. These findings are consistent with our previous observation that no IL-12Rβ2 transcripts were detected in *naive* $CD4^+$ T cells (Fig. 15, left panel).

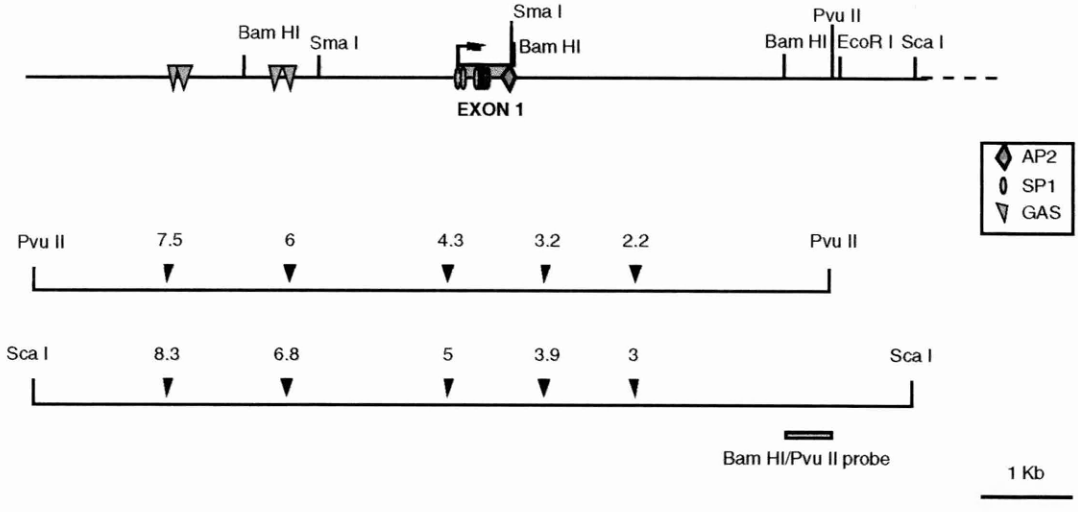

Figure 16. *Mapping of DNase Hypersensitive sites in the IL-12Rβ2 gene.* In this figure are summarised the results obtained by the DHS analysis performed in the 9 kb Pvu II fragment across the IL-12Rβ2 promoter region. The DH sites identified were also confermed analysing a 9.5 kb Sca I fragment, which covers almost the same genomic region contained in the Pvu II fragment. The position of the DNAseI hypersensitive sites (indicated by arrows) with respect to the Pvu II restriction sites in the IL-12Rβ2 gene were defined by the length of the respective subfragment and the position of the BamH I-Pvu II probe.

DHS analysis was then performed on PHA blasts at day 20 after activation. In addition to the parental 9 kb fragment, five subbands were detected in the Pvu II

digestion (Fig. 15, right panel). The DH sites identified were also confirmed in a 9.5 kb Sca I digestion fragment, which covers almost the same genomic region contained in the Pvu II fragment (Fig. 16). A strong DH site mapped in close proximity to the RNA start site, confirming the presence of a core promoter. Putative enhancer regions were identified by two additional DH sites located upstream the RNA start site, a strong one at -3000 and a weaker one at -1900. Two weak DH sites were also mapped in the first intron, at +1100 and at +2100.

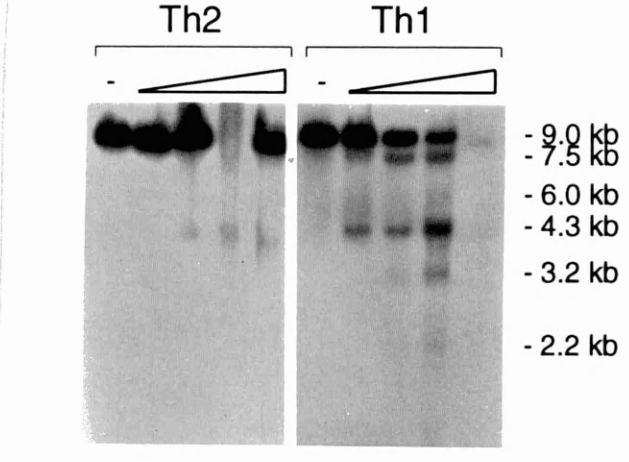

Figure 17. *DNase Hypersensitive sites are present in the IL-12Rβ2 gene in Th1, but not Th2 cells.* Nuclei were purified from Th1 (left panel) or Th2 (right panel) cells at day 6 after priming and treated with increasing amounts of DNase I (lanes 2-5) for 2'. Genomic DNA was extracted, 10 μg were digested with Pvu II and Southern blot analysis was performed using the 0.5 kb Pvu II-BamH I genomic fragment as a probe.

Next, the DHS pattern of Th1 and Th2 cell lines was investigated. Th1 cells analysed at day 6 after priming showed a DHS pattern almost identical to the one observed with PHA blasts (Fig. 17, left panel). Conversely, Th2 cells showed only

one DH site corresponding to the RNA start site (Fig. 16 and Fig. 17, right panel), indicating a poor transcriptional activity of the IL-12Rβ2 gene in these cells, as expected from the previous findings. To test the effects of IL-12 and α-CD3 on the chromatin structure of Th1 cells, lines were rested until day 20 after priming and then stimulated with α-CD3 mAb or IL-12. Both treatments resulted in an increased intensity of the sub-fragments, suggesting that these sites could indeed contain responsive elements for IL-12 and α-CD3 mediated regulation of the gene (data not shown).

3.2.4) Identification of the minimal promoter region

DHS analysis as well as RNA start site mapping evidenced the presence of a promoter region, positioned at 2.5 kb from the 5' end of pBET-9. In order to verify and to characterise this potential promoter region, restriction fragments containing this region were clone upstream to a luciferase reporter gene (pXP; (de Wet et al., 1987)) and used for transient transfections in a number of lymphocytic cell lines. CEM and Jurkat T cell lines were electroporated (or transfected using DMRIE-C) and luciferase activity was detected in the cell lysate 24 hours after transfection. Transfection efficiencies were normalised with β-galactosidase values.

As shown in figure 18, the pXP-23 construct, containing the 700 bp Dra II-Sma I fragment, (covering the RNA start site) was found to be constitutively active in T cell lines, showing 60 fold of induction if compared to the empty vector. When pXP-23 insert was cloned in the opposite direction (pXP-24) no luciferase activity was detected. Extension at the 5' of length of the fragments was not further increasing promoter activity in CEM and Jurkat cells (data not shown)

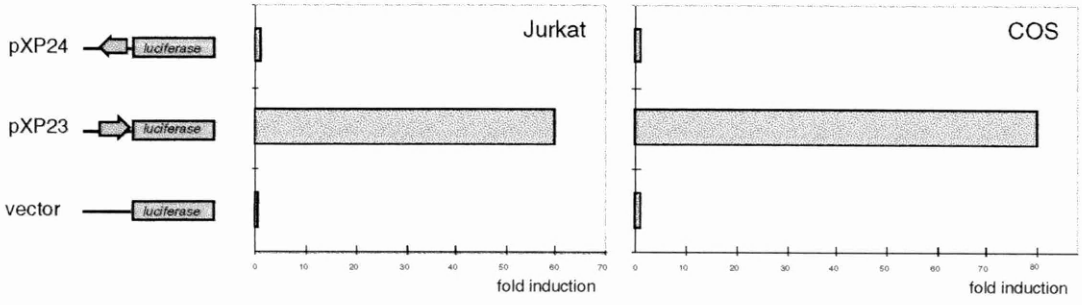

Figure 18. *An IL-12Rβ2 gene fragment of 700 bp activates transcription in Jurkat cells.* the 700 bp fragment Dra II-Sma I of the IL-12Rβ2 gene was cloned in both directions (pXP23 and pXP24) upstream the luciferase gene, in the pXP vector. These constructs were transiently transfected in Jurkat cells (left panel) or COS cells (right panel) and luciferase activity in cell lysates was measured 24 h after transfection. Variability of transfection efficiencywas normalised by measuring β-galactosidase activity of a CMV-gal vector cotransfected in the cells. The results shown are representative of 5 independent experiments.

DNA sequence analysis of this region (see figure 20) revealed the absence of a TATA and a CCAAT box, (Conaway and Conaway, 1993), no initiator sites (Javahery et al., 1994), but the presence of a Multiple start site Element Downstream (MED)-1 element (GCTCC$^{C}/_{G}$) (Ince and Scotto, 1995), consistent with the presence of multiple RNA start sites. In addition, a perfect consensus AP2 binding site (5'-CCGCAGGC-3') and seven potential SP1 binding sites (5'-GGGCGG-3') were identified. These features of the IL-12Rβ2 core promoter, rather unexpectedly, resemble a typical housekeeping gene promoter. Interestingly, transfection of pXP 23 into non-lymphoid cell lines like COS-7 cells (Fig. 18) resulted in promoter activity, suggesting that this region does not confer tissue-specificity to IL-12Rβ2 gene expression.

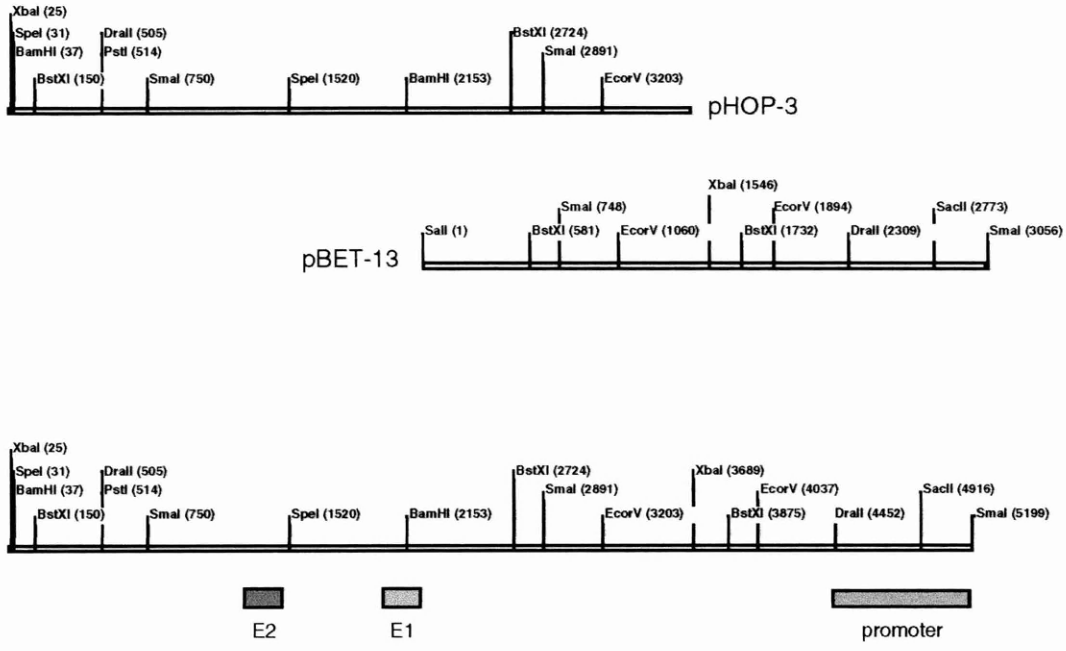

Figure 19. *Restriction map of the IL-12Rβ2 promoter region and of the 4.5 kb upstream.* pBET-13 and pHOP-3 were sequenced as described in materials and methods. The inserts of the two clones partially overlaps. The resulting contig that contains the IL-12Rβ2 promoter and the enhancer regions E1 (that contains β2GAS1 and β2GAS3) and E2 (that contains β2GAS2 β2GAS4) are also shown.
Cutters: BamH I, BstX I, Dra II, EcoR V, Not I, Pst I, Sac I, Sac II, Sma I, Spe I, Xba I & Xma II.
Non-Cutters: Apa I, Bsp106, EcoR I, Hind III, Kpn I, Sal I & Xho I.

Figure 20. *Sequence the IL-12Rβ2 promoter region and of the 4.5 kb upstream region.* pBET-13 and pHOP-3 were sequenced as described in materials and methods. The sequence analysis and the resulting sequence contig was obtained using the Sequencher program. Transcription factor consensus binding sites are indicated.

3.2.5) Identification of IL-12 responsive elements in the IL-12Rβ2 gene

The transcription factors Stat4 and Stat6 have been shown to be essential for T helper development. Stat6 is activated by IL-4 in Th2 cells (Hou et al., 1994; Schindler et al., 1994), whereas IL-12 activates Stat4 in Th1 cells (Bacon et al., 1995; Jacobson et al., 1995). Studies on knockout mice for these transcription factors clearly indicate the pivotal role of Stat6 and Stat4 in T helper cell differentiation. Stat6-deficient T lymphocytes fail to differentiate into Th2 cells in response to IL-4 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996) and the analysis of $Stat4^{-/-}$ T cells revealed an impaired production of IFN-γ upon antigen receptor triggering,indicative of a defect in Th1 differentiation (Kaplan et al., 1996; Thierfelder et al., 1996). More recently, additional transcription factors have been studied and characterized for their role in the Th2-specific expression of the IL-4 gene: the protoncogene c-Maf (Ho et al., 1996), the transcription factors NIP45 (for NF-ATp interacting protein) (Hodge et al., 1996), NF-IL6 (Davydov et al., 1995), and Nuclear Factor of Activated T cells (NF-AT), which has been shown to regulate IL-4 expression in Th2 cells through a cooperative binding with AP-1 family members (Rooney et al., 1995). In addition, the transcription factor GATA-3 has been found to be selectively expressed in differentiating and effector Th2 cells, whereas its expression is completely lost in Th1 cells (Zheng and Flavell, 1997). Less is known about the regulation of IFN-γ gene in Th1 cells that appears to involve the interaction of numerous transcription factors, including NF-kB and NF-AT (Sica et al., 1997), or the cooperative binding of Stat4 dimers (Xu et al., 1996).

In order to understand how the Th1 specific expression of the IL-12Rβ2 gene is controlled, the putative enhancer regions evidenced by DHS analysis upstream the RNA start site at -3000 bp (E2) and -1900 bp (E1) were investigated for transcription factors consensus binding sites.

Sequence analysis of E1 and E2 regions (Fig. 19 and 20) revealed the presence of GAS (Gamma Activated Sequence) consensus binding sites in both regions, defined as $TTCN_{(2-4)}GAA$ (Ivashkiv, 1995) (see materials and methods).

In order to verify whether these sites were binding Stat factors gel shift assays were performed. Three oligonucleotides encompassing a GAS consensus sequence were derived from the IL-12Rβ2 E2 sequence (E2: -3292-3273; E2: -3139-3115; E2: -3029-3005; and three oligonucleotides were designed in the E1 region (E1: -2224-2205; E1: -2158-2139; E1: 1969-1945, see materials and methods). An oligonucleotide representing the GAS element in the promoter of the IRF-1 gene, was used as positive control (Ivashkiv, 1995; Sims et al., 1993).

Gel shifts were performed using TOTEX protein extracts of Th1 and Th2 cell lines at day 5 after priming that had been either unstimulated or stimulated for 1 h with 2.5 ng/ml IL-12. The oligonucleotides E2: -3029-3005 and E1: -1969-1945 (named β2GAS2 and β2GAS1 respectively) and the IRFGAS control formed a DNA-protein complex in IL-12 stimulated Th1, but not in Th2 cells (Fig. 21, lanes 1 and 2, upper and lower panel, respectively).

These findings are consistent with the previous observation that IL-12 signaling in Th2 cells is extinguished (Hilkins et al., 1996; Szabo et al., 1995). Competition with an excess of cold Fcγ GAS oligonucleotide (Fig. 21, lane 3) confirmed Stat specificity. In order to identify which Stat factor caused the observed gel shift, supershift assays were performed using α-Stat1, α-Stat3 and α-Stat4 antibodies. Antibodies against Stat4 completely abolished the formation of the complex, while α-Stat1 and α-Stat3 antibodies did not significantly affect binding to β2GAS1 and β2GAS2 oligonucleotides (Fig. 21, lanes 4, 5 and 6). These data indicated that IL-12 induced binding of activated Stat4 to a potential enhancer elements within the IL-12Rβ2 gene.

To test whether these GAS sites, that showed Stat4 binding capacity, were also able to promote IL-12-inducible transcription, transient transfection experiments were performed.

Many lymphoid cell lines were tested for their ability to respond to IL-12 and their amenability to transfection using the IRF-luc vector. This construct was obtained by cloning 3 copies of the IRFGAS oligonucleotide upstream the SV 40 promoter

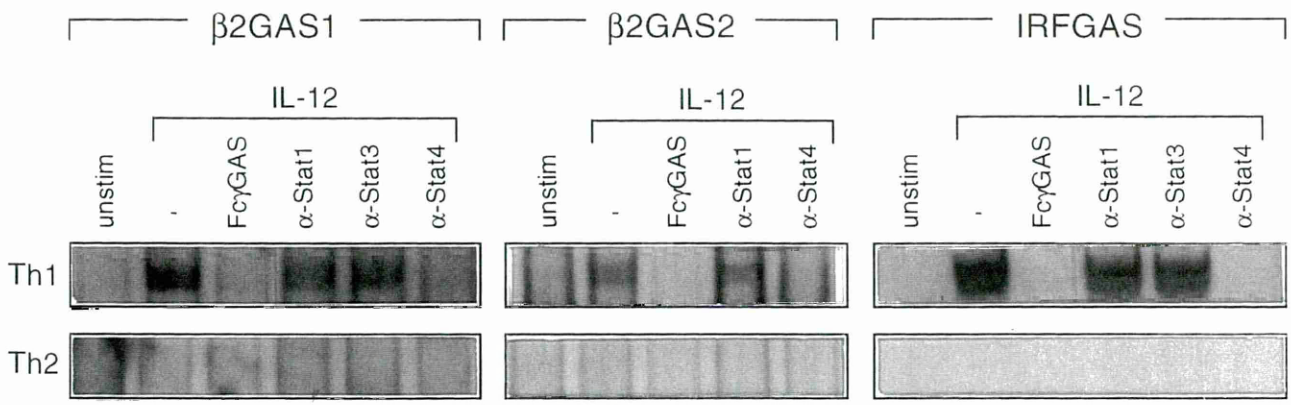

Figure 21. *IL-12 induces Stat4 binding to IL-12Rβ2 GAS sites in Th1, but not Th2 cells.* Th1 and Th2 cells were harvested 5 days after priming, washed and resuspended in complete medium. 10^7 cells were incubated at 37 °C in 4 ml complete medium with or without IL-12 (2.5 ng/ml) for 1h, followed by preparation of whole cell extracts as described in Material and Methods. Gel shifts were performed with ^{32}P end-labeled double stranded oligonucleotides, comparing β2GAS1, β2GAS2 and IRFGAS oligonucleotides binding pattern. Competition with an excess of cold Fcγ GAS oligonucleotide confirmed Stat specificity. In order to identify which Stat factor caused the observed gel shift, supershift assays were performed using α-Stat1, α-Stat3 and α-Stat4 antibodies as described in Materials and Methods.

of the pGL-pro vector. All cell lines tested (see materials and methods Tab. V) showed no responsiveness to IL-12 and thus couldn't be used for this study. To overcome this problem, IL-12 responsiveness was reconstituted in Jurkat or 3 DO cells by cotransfecting all the components of the IL-12 signaling machinery missing in Jurkat and 3DO cells: the two IL-12 receptor subunits (pEF-BOS-β1 and pEF-BOS-β2) and Stat4 (pBOST4).

Two copies of β2GAS1 (see Appendix 3), were cloned in the pGL-pro vector, upstream the SV40 promoter, obtaining the 1DIM-luc construct. 1DIM-luc was transiently cotransfected in Jurkat cells together with pEF-BOS-β1, pEF-BOS-β2 and pBOST4. After electroporation, cells were stimulated for 24 h with IL-12 and luciferase activity measured in the cell lysate (Fig. 22). As a control, the IRF-luc construct was also tested. Variability of transfection efficiency was normalised by measuring β-galactosidase activity of a CMV-βgal vector cotransfected in the cells. Transfections were repeated at least three times. 1DIM-luc constructs lead to a 3 fold induction of luciferase activity when transfected cells were stimulated with IL-12 (Fig. 22, middle panel). When pEF-BOS-β2 was not transfected in the cells, the treatment with IL-12 did not result in any increase in the luciferase activity of the GAS-dimer constructs. Similar experiments were also performed using 2-DIM-luc construct, containing two copies of the β2GAS2 site, obtaining comparable results. These data evidenced that not only the β2GAS1 and β2GAS2 elements found in the IL-12Rβ2 enhancers binds Stat4, but also that they are transcriptionally active and furthermore that their activity is IL-12 dependent. These data provide strong evidence that Stat4 plays a key role in the regulation of the Th1-specific expression of IL-12Rβ2.

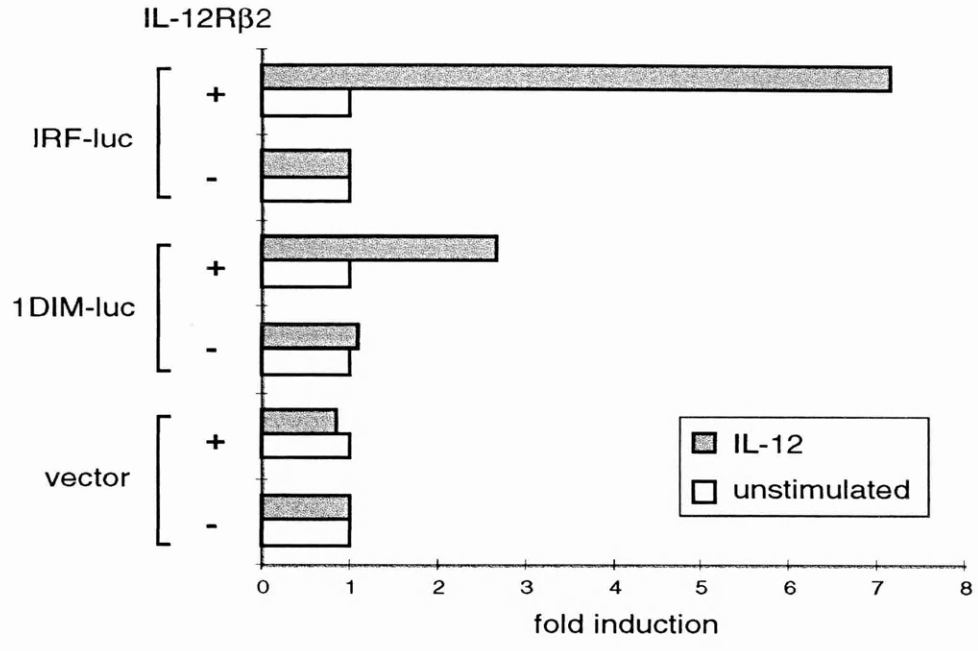

Figure 22. *IL-12 dependent activation of IL-12Rβ2 GAS elements requires a functional IL-12 receptor.* The ability of 1DIM-luc construct, that contains 2 copies of the β2GAS1 oligonucleotide, to be activated in an IL-12 dependent manner was tested. Jurkat cells were transiently cotransfected with the 1DIM-luc (middle panel) or the positive control IRF-luc (upper panel) or the vector alone (lower panel) together with: pEF-BOSβ1 and pBOST-4 plus or minus pEF-BOSβ2 (as indicated). Cells were either left untreated or stimulated for 20 h with IL-12. Luciferase activity was measured 24 h after transfection. Variability of transfection efficiency was normalised by measuring β-galactosidase activity of a CMV-gal vector cotransfected in the cells.

It has recently been described that dimers of dimers of Stat4, can cooperatively bind tandem DNA sequences, stabilising the interaction with "weak" GAS sites (Vinkermeier et al., 1996; Xu et al., 1996). Thus the GAS like sequences, that were not showing binding to Stat factors in single copy, were also tested as dimers. E2:-3139-3115 dimers and E1:-2224-2205 dimers (that I will call from now on β2GAS4 and β2GAS3 respectively) formed a week DNA-protein complex in the presence of IL-12. This complex is abolished by pretreatment with α-Stat4 antibodies (data not shown). These data suggest that also these GAS sequences, despite of the fact that I did not detect binding of Stat factors when tested as single sites in gel shift assays, could participate to Stat binding in association with other GAS sites present in the IL-12Rβ2 gene.

To verify this hypothesis the oligonucleotides β2GAS2 and β2GAS1 were cloned either alone (obtaining the B2-21-luc construct) or together with the β2GAS3 and β2GAS4 oligos (B2-luc) in the pGL-pro vector and compared for IL-12 inducibility in transient transfection assays (Fig. 23).

An IL-12 dependent 3 fold induction of luciferase activity was detected when B2-21-luc construct was tested, showing a comparable activity to the previously characterised 1DIM-luc and 2DIM-luc vectors (Fig. 23). Interestingly, the B2-luc construct, that contains the β2GAS3 and β2GAS4 oligos in addition to β2GAS2 and β2GAS1, showed an even higher induction of luciferase activity in response to IL-12 (Fig. 23). This indicated that the β2GAS3 and β2GAS4 sites can contribute to transcriptional regulation of the IL-12Rβ2 gene when associated to other GAS binding sites.

The E1 and E2 genomic regions of the IL-12Rβ2 gene containing the GAS sites characterised so far were also tested for enhancing activity in response to IL-12.

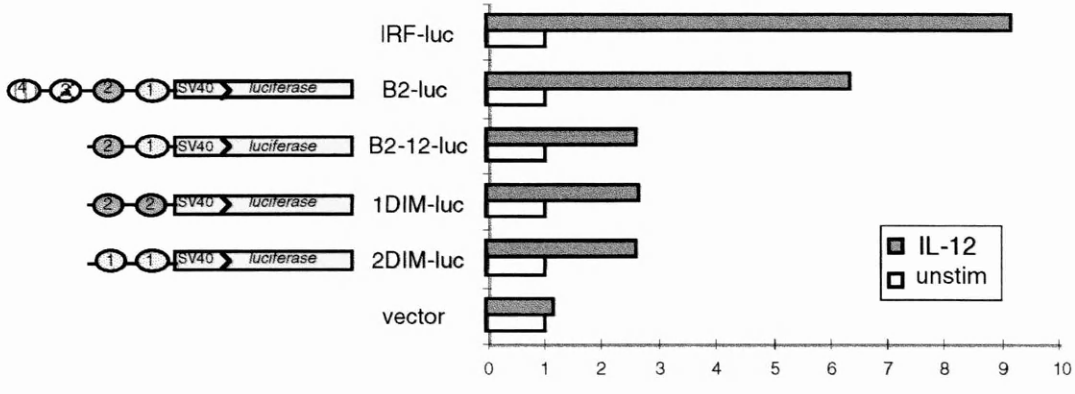

Figure 23. *β2GAS3 and β2GAS4 sites can contribute to transcriptional regulation of the IL-12Rβ2 gene when associated with other GAS binding sites.* The oligonucleotides β2GAS2 and β2GAS1 were cloned either alone (obtaining the B2-21-luc construct) or together with the β2GAS3 and β2GAS4 oligos (B2-luc) in the pGL-pro vector. Ability to promote luciferase transcription in response to IL-12 was tested and compared to the 1DIM-luc, 2DIM-luc and IRF-luc in transient transfection assays.

The Xba I 3.5 kb fragments derived from pHOP-1 and PCR fragments encompassing the regions between -2368-1858 and -3326-2849 were cloned in pGLpro (see Mat. & Met.), and the new constructs were tested in transient transfection experiments. None of the constructs analysed showed IL-12-mediated upregulation of luciferase activity (data not shown). These results could be attributed to a not sufficient sensitivity of the experimental system, since it requires the coincident transfection of 4 constructs in a cell to reconstruct IL-12 signaling.

3.2.6) Discussion

The DNase Hypersensitive Site analysis allowed the characterisation of chromatin structure and accessibility of the IL-12Rβ2 locus in *naive* and developing Th1 and Th2 cells.

The DHS analysis of a 9 kb fragment encompassing the RNA start site in developing T helper cells evidenced marked differences in the chromatin configuration of Th1 and Th2 . Early developing Th2 cells showed a relatively "closed" chromatin configuration with only one DH site in the proximity of the RNA start window.

Conversely, Th1 cells displayed an accessible chromatin configuration with a complex pattern of DNase Hypersensitivity comprised of 5 DH sites: two strong DHS at the RNA start region and at -3000, and a weaker one at -1900; two weak DH sites were also mapped in the first intron, at +1100 and at +2100. The same hypersensitivity pattern was also evidenced in PHA blasts activated in the presence of IL-12.

The differences in IL-12Rβ2 locus accessibility evidenced in primary Th1 versus Th2 cells could result from selective repression of the IL-12Rβ2 locus in Th2 cells or from selective activation of the locus in Th1 cells. The previous finding that no IL-12Rβ2 transcripts were present in *naive* $CD4^+$ T cells would support the second hypothesis. Indeed, DNase I Hypersensitivity analysis of purified $CD4^+$

naive T cells demonstrated that these cells displayed a chromatin configuration correspondent to a transcriptionally incompetent locus, exhibiting a very faint, if any, DH site. This site maps in the proximity of the RNA start window and it would be consistent with the presence of a paused RNA Polymerase II complex, as previously described for DH sites in the first intron of the IL-2 and the c-*myc* genes (Bentley and Groudine, 1986; Brunvand et al., 1993). Polymerase occupancy of resting genetic loci would allowed rapid gene transcription upon stimulation. Indeed IL-12Rβ2 transcripts can be detected as early as 6 h after TCR induced activation of *naive* T cells (see chapter 1).

The same DH site is also the only one observed in Th2 cells. Yet, this is again consistent with our previous observation (chapter 1) that small amounts of IL-12Rβ2 transcripts could be detected early on along differentiation. These data could be interpreted as an initial wave of IL-12Rβ2 gene transcription still taking place in Th2 cells after the initial T cell activation, that is extinguished soon afterwards. On the other hand, it could not be excluded an heterogeneity of the Th2 cell line that could contain small amounts of Th1/Th0 cells, in which the IL-12Rβ2 gene is transcribed.

The analysis of the hypersensitive site at the RNA start window, allowed the characterisation of the minimal promoter region. Promoters transcribed by RNA polymerase II are divided in two classes: those that contains a canonical TATA box and those that do not. TATA containing promoters usually direct transcription from a single initiation point, the location of which is determined by the TATA position (Conaway and Conaway, 1993). In TATA-less promoters start site selection is not well understood and has been investigated primarily in genes that use a single transcription start site, where the presence of an "initiator" element, at or near the start site, appears to be responsible for localising the pre-initiation complex (Javahery et al., 1994). Recently a new class of TATA-less promoters has been described that utilises a distinct window of multiple start sites defined by the presence of a Multiple start site Element Downstream (MED)-1 element (Ince and

Scotto, 1995). The IL-12Rβ2 appears to belong to this last class of promoters, since a perfect consensus MED-1 site can be found 152 nucleotides downstream the RNA start window of 30 nucleotides.

The minimal promoter contains a CpG rich region in which multiple recognition motifs for the transcription factor SP1 can be found. These GpC rich boxes have been described to be required for efficient transcription of TATA-less promoters (Blake et al., 1990; Kollmar et al., 1994). These characteristics of the IL-12Rβ2 promoter resembles in structure that of many housekeeping genes, but IL-12Rβ2 gene is expressed in a tissue specific manner (Chua et al., 1994; Chua et al., 1995; Desai et al., 1992). Indeed, few GC-rich and TATA-less promoters that show a tissue specificity were also described, such as the promoters of the Transcription Enhancer Factor (TEF)-1 (Boam et al., 1995) and the nerve growth factor receptor gene (Sehgal et al., 1988). Interestingly, the IL-12Rβ2 minimal promoter is equally active in lymphoid (Jurkat, CEM, NK3.3) and non-lymphoid (COS-7) cells. Several reasons may account for the apparent inability of the IL-12Rβ2 promoter to function in a tissue-specific manner *in vivo*, but not *in vitro*. Tissue specificity could be determined by selective downregulation of the promoter accessibility by CpG methylation. If this was the case, transient transfection experiments would not reveal this mechanism since the DNA amplified in bacteria is almost unmethylated. Alternatively, tissue-specific *cis*-acting sequences could be localised elsewhere. Also a selective degradation of the IL-12Rβ2 mRNA could occur, but this is very unlikely, since analysis of IL-12Rβ2 transcripts in the presence of the RNA synthesis inhibitor Actinomicin D, didn't evidence post-transcriptional regulatory mechanism (data not shown).

Four GAS consensus binding sites were identified in the region upstream the minimal promoter. Gel shift assays revealed that two of them, β2GAS1 and β2GAS2, bind to IL-12 induced Stat4. Despite of the fact that also Stat3 phosphorylation has been described to occur in response to IL-12 (Szabo et al., 1995), anti-Stat3 antibodies did not significantly change the IL-12 induced DNA-

binding complexes suggesting that Stat3 does not play a major role in activating the IL-12Rβ2 promoter in response IL-12. Transient transfection experiments in Jurkat cells demonstrated that the β2GAS1 and β2GAS2 elements found in the IL-12Rβ2 enhancers not only bind Stat4, but also that they are transcriptionally active and furthermore that their activity is IL-12 dependent. These data provide strong evidence that Stat4 plays a key role in the regulation of the Th1-specific expression of the IL-12Rβ2 gene.

The other two GAS consensus sequences β2GAS3 and β2GAS4 were not binding any Stat factor in single copy, but this does not exclude the involvement of these sites in IL-12 mediated regulation of the IL-12Rβ2. Dimers of dimers of Stat4 cooperatively bind to "weak" GAS sites, as described in the IFN-γ gene (Vinkermeier et al., 1996; Xu et al., 1996). As a matter of fact, dimers of β2GAS3 and β2GAS4 bind to Stat4 (data not shown). Furthermore, a luciferase reporter construct containing all four IL-12Rβ2 GAS showed an increased upregulation in response to IL-12 if compared to the construct that contains only the β2GAS1 and β2GAS2. These finding suggest that also β2GAS3 and β2GAS4, could contribute to IL-12Rβ2 regulation.

Figure 24. *IL-12Rβ2 GAS sites binding specificity*

		Binding to Stat4	Binding to Stat1
STAT4 consensus	$^{T}/_{A}$ TTC $C^{G}/_{C}G$ GAA$^{T}/_{A}$,		
IRFGAS	T TTC CCC GAA A	+	+
β2GAS1	T TTC CTT GAA A	+	-
β2GAS2	T TTC CTA GAA A	+	+/-
β2GAS3*	A TTC ATA GAA T	+	+/-
β2GAS4*	A TTC TCA TAA T	+/-	-

IL-12Rβ2 GAS sites binding specificity. Nucleotides in the IL-12Rβ2 GAS sites that satisfy Stat4 consensus binding sequence are underlined. Binding to Stat4 and Stat1, determined by gel shift assays, is also indicated.

* β2GAS3 and β2GAS4 binding data are obtained performing gel shift with dimers

The four IL-12Rβ2 GAS sites are located far from the minimal promoter, and are also distant one from the other. *The shortest distance between two GAS elements is 100 nucleotides. The attempt of demonstrating IL-12 mediated activation by these sites directly testing a 3.5 kb region containing all four GAS sites or shorter genomic regions in transient transfection assays was unsuccessful. These constructs might be inefficient in promoting transcription either because they contain silencing regions that can mask Stat4 mediated activation or alternatively because they cannot reproduce the correct chromatin conformation. Another possibility is that these results could be attributed to a not sufficient sensitivity of the experimental system. Indeed, the reconstitution of an IL-12 responsive cell line required the coincident transfection of 4 constructs in Jurkat cells, which is not the most efficient tool to test IL-12 responsiveness. The best cell types in which IL-12Rβ2 regulation should be ideally analysed are Th1 and Th2 lines, but these primary T cells are not efficiently transfectable. Indeed, much more efficient retroviral vectors are now substituting the transfection techniques (Dumbar and Young, 1996). The problem of using retroviral vectors in analysing the regulation of a promoter is that the strong retroviral promoters will interfere with the one under investigation. Self-inactivating retroviral vectors, potentially useful for promoter characterisations have also been developed, but they still present numerous limitations (Nakajima et al., 1993).

Yet some laboratories reported successful electroporation protocols (Hughes and Pober, 1996) that I tried to reproduce and I tested most of the new liposomes described to mediate transfection of primary non-adhesive cells (see materials and methods), but all these approaches gave too low transfection efficiencies.

Recently, Huang and colleagues (Huang et al., 1998) have been able to detect an IL-4 promoter specific activation in recently-differentiated Th2 helper cells, but this

required antibody-mediated immobilisation of these cells and the use of particle mediated gene transfer.

The transcription factor Stat4 has been shown to be essential for T helper development. The analysis of Stat4$^{-/-}$ T cells revealed an impaired production of IFN-γ upon antigen receptor triggering, indicative of a defect in Th1 differentiation (Kaplan et al., 1996; Thierfelder et al., 1996). Recently, Agarwal and colleagues (Agarwal and Rao, 1998) showed chromatin remodeling of the IL-4 locus along Th2 differentiation. They demonstrated that this does not occur in Stat6 deficient mice, thus indicating that Stat6 is necessary for the "opening" of the IL-4 locus.

Loci that have been repressed can become transcriptionally active following hyperacetylation of histones and demethylation of DNA (Hagstrom and Schedl, 1997; Nan et al., 1998). Furthermore, it has been recently shown that cell cycle progression and cytokine signaling act in concert to relieve epigenetic repression and can be supplanted by agents that hyperacetylate histones and demethylate DNA (Bird et al., 1998).

Stat1 is associated with and requires the action of both CBP/p300 and pCAF (Korzus et al., 1998; Yang et al., 1996). These are transcriptional coactivators that exhibit strong histone acetyltransferase activities (Ogryzko et al., 1996).

It could be very interesting to investigate whether also Stat4 and Stat6 require for their activity the interaction with p300/CBP or similar proteins and whether their acetyltransferase action could be involved in chromatin remodeling along T helper differentiation.

Our findings that Stat4 binds and activates the IL-12Rβ2 locus in response to IL-12 and yet that this locus is remodeled in Th1 but not Th2 cells, suggests a direct role of Stat4, in analogy to Stat6 for the IL-4 loci, in chromatin remodeling of the IL-12Rβ2 locus and thus in imprinting a "memory" which will be maintained through cell division. In this respect, the methylation and acetylation state of the IL-12Rβ2 locus and its chromatin accessibility in Stat4 deficient mice should be investigated.

The data presented in the previous chapter evidenced the importance of stimulation through the antigen receptor for IL-12Rβ2 initial transcription. In this chapter, the DNase I hypersensitivity pattern evidenced that the coordinate action of antigen receptor stimulation and IL-12 results in a transcriptionally active IL-12Rβ2 locus. Which TCR-activated nuclear factors might play a role, in association with Stat4, in locus remodeling of the IL-12Rβ2 gene?

To investigate the role of TCR-activated transcription factors such as NF-AT and NF-kB, the effects of specific pharmacological inhibitors, such as Cyclosporine A and Dexamethasone, were studied, but no striking effects were observed (data not shown). Alternatively, TCR-activated MAP kinase signaling pathways could be involved. Recently, Yang and colleagues (Yang et al., 1998) described that JNK-2 deficient $CD4^+$ T cells fail to differentiate in Th1 cells. IL-12Rβ2 expression in JNK-2 -/- $CD4^+$ T is reduced, but still remains to be elucidated whether this is a direct effect on IL-12Rβ2 gene or whether it correlates with the lower levels of IFN-γ that these cells produce, since IFN-γ was shown to upregulate IL-12Rβ2 in mice (see chapter 4). Also the p38 MAP kinase is involved in IFN-γ gene activation in differentiated Th1 cells, but no differences in its activation are detected among early differentiating Th1 and Th2 cells (Rincon et al., 1998). Yet, the MAP kinases activated transcription factors that are involved in IFN-γ regulation are to be identified, and it would be interesting to test whether they can act on the IL-12Rβ2 gene. Further analysis of the yet uncharacterised Th1 specific DH sites observed in the IL-12Rβ2 locus could provide new insights to clarify this issue.

3.3) Role of IFNs in human T helper development

3.3.1) IFN-α induces IL-12Rβ2 mRNA expression and IL-12 responsiveness in human T cells.

In addition to IL-12, IFN-α has also been shown to influence Th1 cell development. Stimulation of resting human T cells in the presence of IFN-α increases the frequency of IFN-γ secreting $CD4^+$ T cells (Brinkmann et al., 1993) and allergen-specific T cell clones generated in the presence of IFN-α from the peripheral blood of atopic patients demonstrate a shift towards the Th0/1 phenotype (Parronchi et al., 1996). In contrast, IFN-γ but not IFN-α has been shown to be an important co-factor for Th1 development in mice (Bradley et al., 1996; Schmitt et al., 1994). IFN-γ can rescue IL-12Rβ2 mRNA expression even in the presence of IL-4 and restore IL-12 responsiveness in early developing mouse Th2 cells (Szabo et al., 1997). Thus, the effects of IFN-γ on phenotype development and on IL-12Rβ2 mRNA expression in humans were also analysed. The addition of IFN-α (Fig. 25A) as well as IFN-β (data not shown) to human cord blood leukocytes stimulated under Th2-inducing conditions (IL-4 and anti-IL-12 mAb) resulted in cells producing IFN-γ (>1 ng/ml) upon restimulation with anti-CD3 mAb (Fig. 25A). IL-12Rβ2 mRNA was expressed in the IFN-α treated T cell lines (Fig. 25B) and IL-12 induced Stat4 phosphorylation in these cells (Fig. 25C).

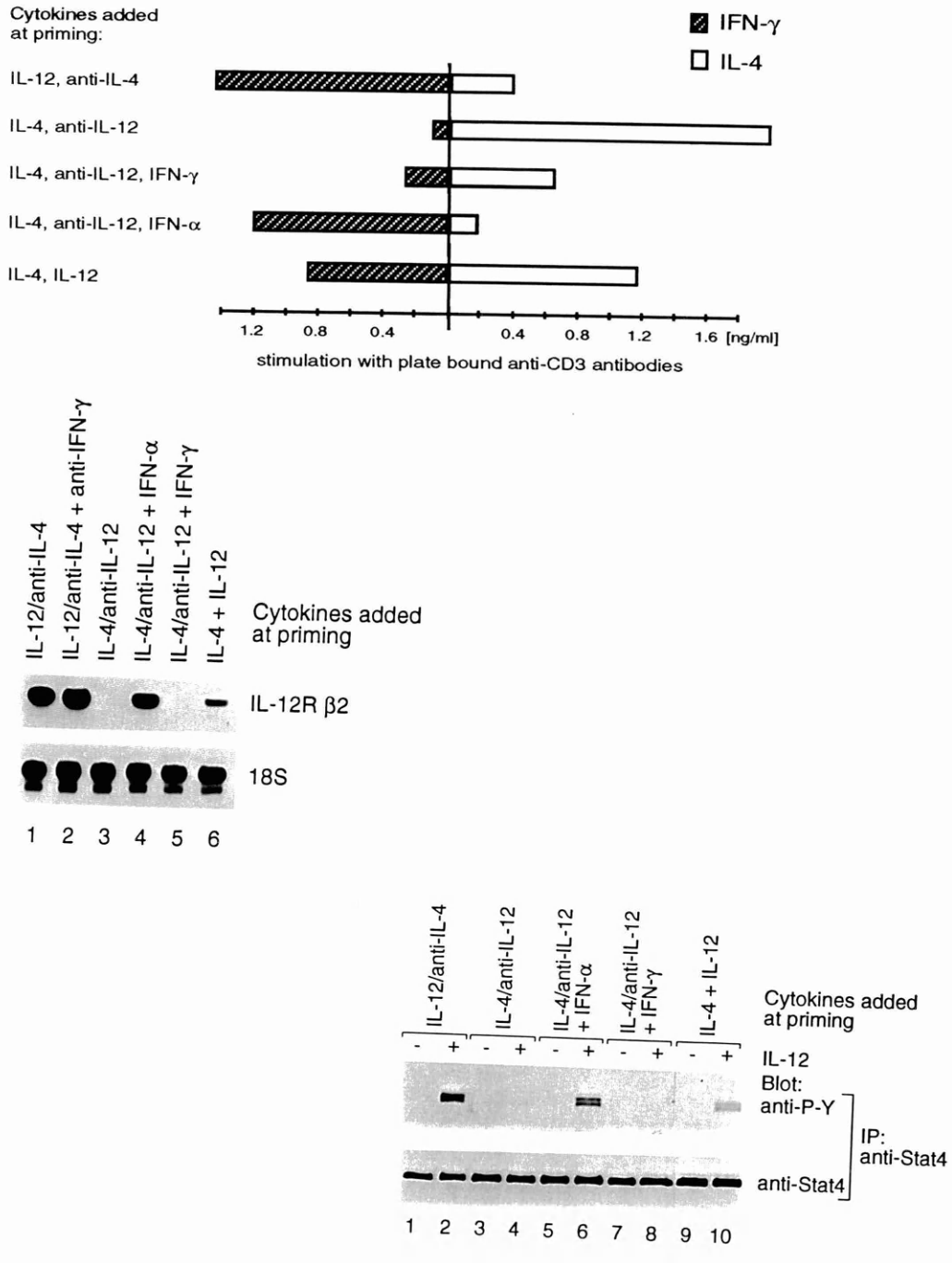

Figure 25. *Function of type I and II interferons in the development of T helper cell subsets and on the expression of the IL-12Rβ2 subunit.* **A)** IFN-a induces Th1 development. T cell lines were generated by stimulating cord blood lymphocytes with PHA in the presence of the indicated cytokines or anti-cytokine antibodies. IFN-a or IFN-g (1000 U/ml) were added at the time of priming to the cultures as indicated. Cytokine production was determined on day 10. IFN-a, but not IFN-g, induces a Th1 phenotype even in the presence of IL-4 and neutralizing anti-IL-12 antibodies. **B)** IFN-a induced Th1 cells express IL-12R b2 transcripts. RNA was extracted from T cell lines 10 days after priming in the presence of the indicated cytokines or neutralizing anti-cytokine antibodies. RNase protection assays were performed as described in Materials and Methods. **C)** IFN-a induced Th1 cells are IL-12 responsive. T cell lines (Fig. 18A) were harvested on day 10 after priming. 8 x 10^6 cells were washed and incubated 15 min at 37 °C in medium with or without 2 ng/ml IL-12 in 1 ml RPMI-FCS. IL-12 induced Stat4 phosphorylation was determined as described above.

Addition of IFN-γ to cultures containing IL-4 and anti-IL-12 (Th2-inducing conditions) or addition of neutralizing anti- IFN-γ mAb at the time of Th1 priming had no significant effect on the pattern of Th cell development (Fig. 25A and data not shown) and slight effects on IL-12Rβ2 expression (Fig. 25B). Both IFN-α or IFN-γ had no significant effect on the expression of the IL-12Rβ1 transcripts (data not shown). These findings suggested that type I and type II IFNs could have different functional effects on human as compared to mouse T cells.

The molecular basis for this species-specificity has been recently investigated by Rogge and coworkers (Rogge et al., 1998). In this study they showed that IFN-α/β are able to induce directly Th1 development, in the absence of IL-12, in human but not in mouse T cells. Conversely, IFN-γ acts in both species in at least two ways: on the one hand IFN-γ induces Th1 development by enhancing IL-12-production by human and mouse phagocytic cells (Trinchieri, 1995), and on the other hand, it upregulates expression of functional IL-12 receptors on $CD4^+$ T cells, but at much higher levels in mouse rather than in human T cells (Rogge et al., 1998).

The findings that IFN-α efficiently induces phosphorylation of Stat4 in mitogen activated PBMC (Cho et al., 1996) and that IFN-α/β phosphorylate Stat4 in human but not in mouse T helper cells (Rogge et al., 1998) provided an explanation for the species specific activity of type I Interferons in human but not mouse T cells. On the contrary, these data did not explain why IFN-γ does not play a significant role in human T helper development. The analysis of Stat binding specificity of the IL-12Rβ2 GAS sites provided new insights in this respect.

3.3.2) Stat binding specificity of the IL-12Rβ2 GAS sites.

The Stat4 binding sequence, determined by binding site selection (Yamamoto et al., 1997), can satisfy also the more general consensus binding for Stat factors (see above). For example, the IRFGAS sequence, which is a perfect for Stat4 recognition sequence, is not Stat4 specific, but binds also Stat1, Stat3, Stat5 and

Stat6 (Ivashkiv, 1995). Therefore, I wondered whether, in addition to Stat4, other Stat factors could bind to the IL-12Rβ2 GAS sites. In particular, Stat1 has been shown to be activated by both type I and type II interferons (Meraz et al., 1996), thus its binding pattern to the IL-12Rβ2 GAS sites was investigated. The analysis of IFN-γ signaling in T helper cells revealed that only Th2 cells resulted responsive to IFN-γ (Bach et al., 1995; Pernis et al., 1995). In order to investigate the binding of Stat1 to IL-12Rβ2 GAS sites, Th2 cells were stimulated with IFN-γ and gel shifts were performed comparing β2GAS1, β2GAS2 and IRFGAS oligonucleotides binding pattern (Fig. 26 lower panel). IL-12 induced binding of Stat4 to these oligos in Th1 extracts was also performed as a control (Fig. 26 upper panel).

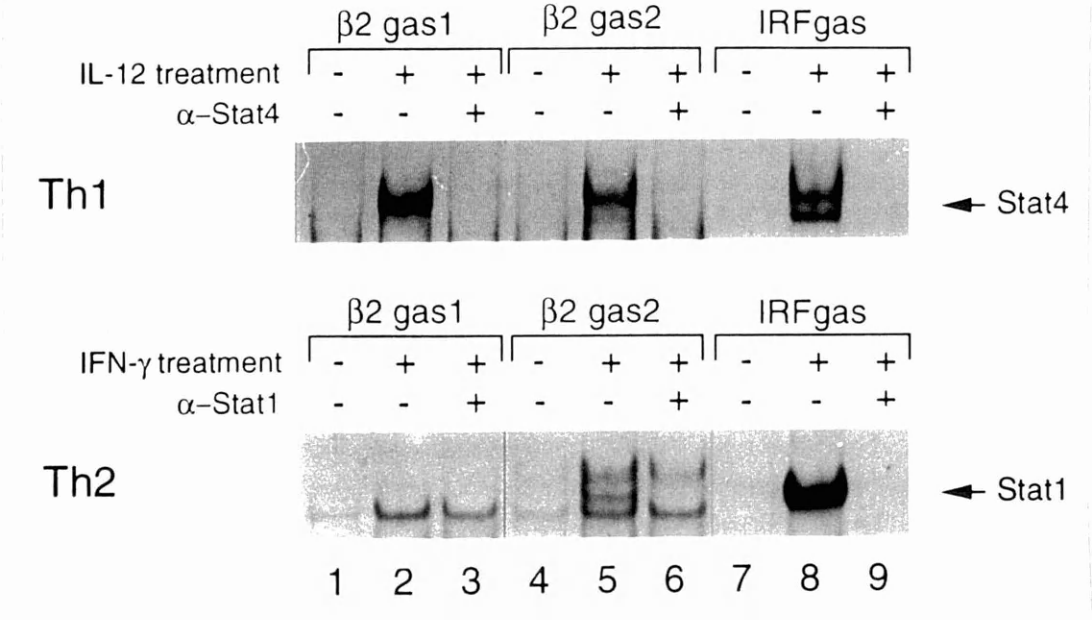

Figure 26. *IL-12Rβ2 GAS sites shows a binding specificity for Stat4.* To test whether Stat1 binds to IL-12Rβ2 GAS sites, Th2 cells were stimulated with IFN-γ and gel shifts were performed comparing β2GAS1, β2GAS2 and IRFGAS oligonucleotides binding pattern (lower panel). IL-12 induced binding of Stat4 to these oligos in Th1 extracts was also performed as a control (upper panel). Supershifts with anti-Stat4 and anti-Stat1 antibodies evidenced that the IL-12- and IFN-γ–induced complexes contained Stat4 and Stat1 respectively (lanes 3, 6 and 9).

As previously shown, Stat4 binding was observed with all 3 oligonucleotides tested, whereas no IFN-γ-induced complexes were detected in the presence of β2GAS1 oligo (Fig. 26, lower panel, lane 2), while β2GAS2 showed a very faint band if compared to the one observed for the IRFGAS oligonucleotide (Fig. 26, lower panel, lanes 5 and 8). The IFN-γ induced complex formed with the IRFGAS probe contained Stat1, as shown by supershifts with anti-Stat1 antibodies (Fig. 26, lower panel, lane 9). These results demonstrated a scarce of even absent interaction of Stat1 with the IL-12Rβ2 GAS sites. In order to further investigate this issue, competition assays between β2GAS1 and IRFGAS in binding to Stat1 or to Stat4 were performed, as shown in Fig. 27. Labeled IRFGAS probe was competed by a 10- or 50-fold molar excess of unlabeled β2GAS1 oligonucleotide (Fig. 27, lanes 3 and 4) or of unrelated control oligonucleotide (SRF, Fig. 27, lanes 7 and 8), with nuclear extracts from IFN-γ-treated-Th2 cells in order to detect Stat1 binding (Fig. 27, lower panel) or IL-12-treated Th1 cells to reveal Stat4 binding (Fig. 27, upper panel).

The radioactivity of the bands of the resulting DNA-protein complexes was quantified in a Molecular-imager and the percentage of binding inhibition of IRFGAS to the Stats was calculated. A 10 fold molar excess of unlabelled β2GAS1, does not significantly modify IRFGAS binding to Stat1 in IFN-γ-stimulated Th2 cells (Fig. 28, lower panel, lanes 3 and 4), whereas the same amount of unlabelled β2GAS1 reduced 50 % of the IL-12 induced IRFGAS binding to Stat4 in Th1 cells (Fig. 28, upper panel, lanes 3 and 4). These experiments confirmed that the binding of IL-12-induced Stat4 to GAS elements in the enhancers of the IL-12Rβ2 gene is much stronger than binding of IFN-γ-induced Stat1 and provide first evidence for selective binding of Stat4 to specific GAS elements.

I next analysed whether this binding selectivity correlates with the observed failure of IFN-γ to significantly induce IL-12Rβ2 gene expression in human T cells (see above). The transcriptional activity of the IL-12Rβ2 GAS elements in response to

IL-12 and IFN-γ was determined. The B2-luc construct, containing all four IL-12Rβ2 GAS sequences, was compared to the IRFluc construct for responsiveness to IL-12 and IFN-γ mediated transcriptional activation in transient transfection experiments. Jurkat cells were transiently cotransfected as described before to reconstitute IL-12 signaling and with the test construct and either left untreated or stimulated for 20 h with IL-12. Luciferase activity was measured 24 h after transfection. B2-luc and IRF-luc were almost equally induced in response to IL-12.

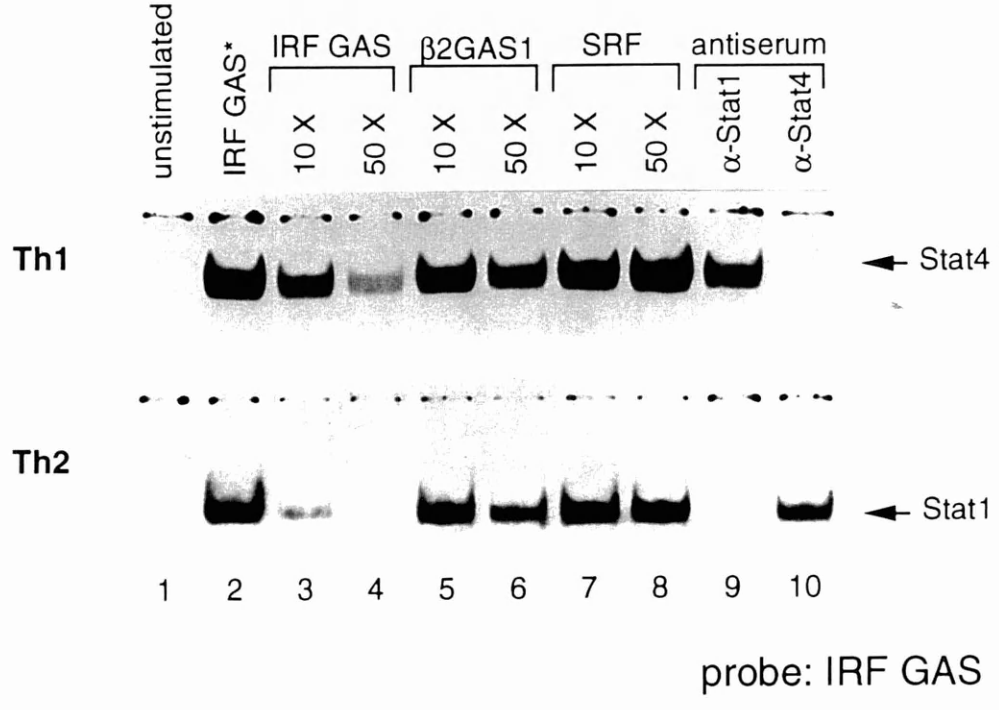

Figure 27. *The β2GAS1 oligonucleotide competes binding of Stat4, but not of Stat1, to the IRFGAS oligonucleotide.* Competition assays between β2GAS1 and IRFGAS in binding to Stat1 or to Stat4 were performed. Labeled IRFGAS probe was competed with a 10- or 50-fold molar excess of unlabeled β2GAS1 oligonucleotide (lanes 3 and 4) or an unrelated control oligonucleotide (SRF, lanes 7 and 8), with nuclear extracts from IFN-γ-treated-Th2 cells in order to detect Stat1 binding (lower panel) or IL-12-treated Th1 cells to reveal Stat4 binding (upper panel). The radioactivity of the bands of the resulting DNA-protein complexes was quantified with a Molecular-imager and the percentage of binding inhibition of IRFGAS to the Stats was calculated.

A 7 fold induction was observed for B2-luc and a 9 fold induction was detected with the IRF-luc (Fig. 28, left panel). IFN-γ mediated induction of the B2-luc and

the IRF-luc was also analysed in transiently transfected Jurkat cells. B2-luc gives a response to IFN-γ that is 10 times lower than the one observed for the IRFluc construct (Fig. 28, right panel). These data demonstrated that the binding selectivity displayed by the IL-12Rβ2 GAS sites is functionally significant and shared by all the GAS sites identified.

In conclusion, the finding that Stat1 does not bind efficiently to the IL-12Rβ2 enhancer regions provides an explanation for the observation that IFN-γ does not contribute to IL-12Rβ2 subunit upregulation and consequently to Th1 development in human T cells (Rogge et al., 1997; Rogge et al., 1998; Wenner et al., 1996). In addition, these data suggest that IFN-α-induced IL-12Rβ2 expression and Th1 development are mediated by Stat4.

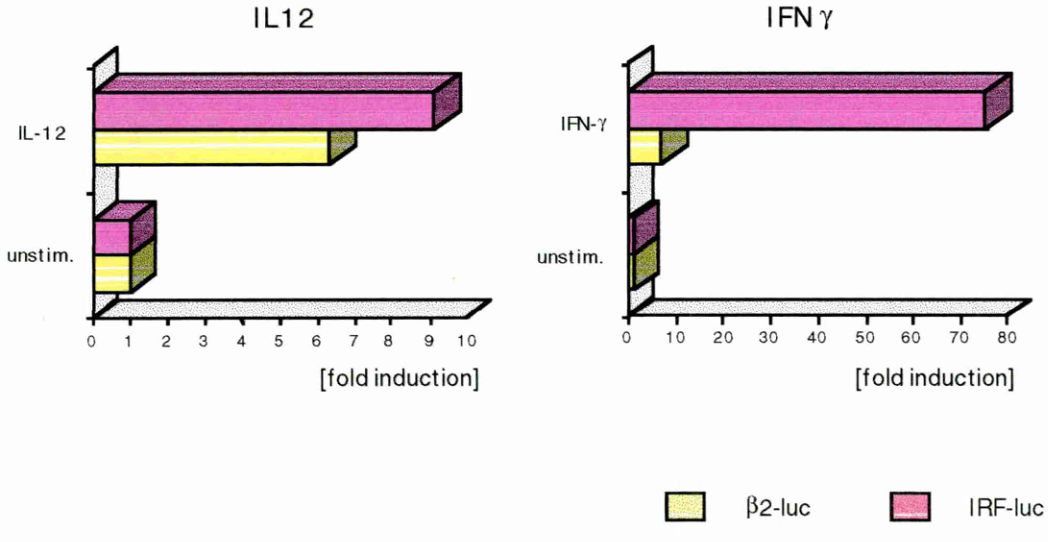

Figure 28. *IL-12Rβ2 GAS elements inefficiently mediate IFN-γ induced transcriptional activation.* The B2-luc construct, containing all four IL-12Rβ2 GAS sequences, was compared to the IRF-luc construct for responsiveness to IL-12 and IFN-γ mediated transcriptional activation in transient transfection experiments. *Left panel*: Jurkat cells were transiently cotransfected as described in materials and methods to reconstitute IL-12 signaling plus the test construct and either left untreated or stimulated for 20 h with IL-12. Luciferase activity was measured 24 h after transfection. *Right panel*: Jurkat cells were transfected with the B2-luc or IRF-luc constructs and stimulated for 20 h with IFN-γ. Luciferase activity was measured 24 h after transfection. All transfections were performed in triplicates and this experiment is representative of three independent ones. Variability of transfection efficiency was normalised by measuring β-galactosidase activity of a CMV-gal vector cotransfected in the cells.

3.3.4) Discussion

The functions of IFN-α/β and IFN-γ in inducing differentiation of human and mouse *naive* T cells to Th1-type effector cells have remained controversial.

Some previous findings in the human system suggested a role of IFN-α in T helper 1 development (Brinkmann et al., 1993; Parronchi et al., 1996). In contrast, IFN-γ, but not IFN-α, has been shown to be an important cofactor for Th1 development in mice (Bradley et al., 1996; Wenner et al., 1996). Thus the effects of IFN-α/β and IFN-γ in T helper differentiation were investigated. The data presented in this chapter demonstrate that the addition of type I interferons to human cord blood leukocytes, in the absence of IL-12, leads to the development of IFN-γ producing Th1 cells. It is interesting to note that IFN-α seems to dominate over IL-4, since IFN-α induced Th1 development takes place even in Th2- inducing conditions. In contrast, addition of IFN-γ to cultures containing IL-4 and anti-IL-12 (Th2-inducing conditions) or addition of neutralizing anti- IFN-γ mAb at the time of Th1 priming had no significant effect on the pattern of Th cell development. Importantly, the effect of IFN-α on T cells is direct, as it occurs in the absence of antigen presenting cells or exogenous factors other than IFN-α/β (Rogge et al., 1998). Together, these findings provide clear evidence that human Th1-type immune responses can develop in the absence of IL-12.

Like in IL-12 induced Th1 development, interferon induced development is strictly correlated with the expression of the IL-12Rβ2 gene. As shown in this chapter, IFN-α induces IL-12Rβ2 mRNA and the consequent development of IL-12-responsive human cells, while IFN-γ only weakly increases IL-12Rβ2 transcription. In analogy to the IL-12 induced Th1 development, IFN-α effects are mediated by Stat4 (Rogge et al., 1998).

In parallel, Szabo and colleagues observed that IFN-γ can rescue IL-12Rβ2 mRNA expression even in the presence of IL-4 and restore IL-12 responsiveness in early developing mouse Th2 cells (Szabo et al., 1997).

Thus, IFN-γ acts indirectly on Th1 development in both species by enhancing IL-12-production by human and mouse phagocytic cells (Trinchieri, 1995), but its direct influence on T helper development can be evidenced in mouse cell only, since it regulates IL-12 receptor expression on $CD4^+$ at much higher levels in mouse rather than in human T cells (Rogge et al., 1998). However, these data do not explain is why IFN-γ does not efficiently act in human Th1 development as it does in mice.

The analysis of Stats binding to the human IL-12Rβ2 GAS sites revealed that Stat1 does not efficiently bind the IL-12Rβ2 GAS sites, while Stat4 binds to them and mediates IL-12 induced upregulation of the gene, as previously described (chapter 2).

In addition, transient transfection experiments showed that the binding selectivity displayed by the IL-12Rβ2 GAS sites is functionally significant and shared by all the B2-GAS sites. The comparison of IL-12 and IFN-γ induced activation of the B2-luc and IRF-luc constructs showed a reduced responsiveness of B2-luc to IFN-γ, if compared to the IRF-luc, while a comparable responsiveness to IL-12 is displayed by both constructs.

Thus, the weak effects exerted by IFN-γ in humans on IL-12Rβ2 gene regulation, and the consequent failure to induce Th1 development, can be explained by the inefficient binding of the IFN-γ -induced Stat1 to the IL-12Rβ2 GAS sites. In this respect it would be interesting to investigate the binding specificity of the mouse IL-12Rβ2 promoter region and to analyse whether the binding specificity of the GAS sites present in the mouse gene is different.

The issue of Stat binding specificity to GAS sites is of great interest to answer the more general question of how Stats accomplish specific signaling from receptors to their target genes. The IL-12 signaling pathway represent a good example of how fine signaling modulation is obtained despite a limited repertoire of available Stat proteins.

A first level of specificity is realised at the receptor level, where it transmits the specificity of the ligand-receptor interaction into the cell. Stat4 activation is strictly tuned in a cells specific way through the selective expression of functional IL-12R on T and NK cells. The picture is even more complicated than this, since Stat4 was shown to be phosphorylated in response to IL-2 in NK cells, but not in T cells, and this cell-specificity was correlated to the ability of IL-2 to phosphorylate uniquely Jak2 in NK cells (Wang et al., 1999). Conversely, this kinase is activated in T cells after IL-12 or IFN-α treatment. IFN-α mediated activation of Stat4 occurs in a species-specific way. The differential signalingcan be also determined by different receptor complexes recognising the same cytokine, for example IL-12 act on DC cells promoting NF-kB nuclear localisation and this has been correlated to the present of a different form of the IL-12Rβ1 subunit (Grohmann et al., 1998). The end point of the signaling cascade and thus the final level of specificity, resides in the induction of genes. Differences in the nature of the GAS elements in the promoters of genes may be a way to obtain specificity. The optimal binding sites of the different Stats, determined by immunoprecipitation and PCR amplification, evidenced a common consensus sequence: $TTCN_3GAA$ (O'Shea, 1997), with the exception of Stat6 that recognises also $TTCN_4GAA$ (Kohler and Rieber, 1995; Schindler et al., 1994). The analysis of the natural binding sites evidenced that some DNA sequences indiscriminately bind all Stat, such as the GAS sequence present in the IRF-1 promoter utilised in this study. Few examples of Stat specificity were also described: the optimal binding sites for Stat1 and Stat3 are identical, yet studies with natural GAS sites have demonstrated different affinities for Stat dimers (Horvath et al., 1995; Rothman et al., 1994). The GAS site present in the serine protease inhibitor (SPI) promoter binds Stat5, but not Stat1 or Stat3 (Wood et al., 1997). The data presented in this chapter provide a first evidence for selective binding of Stat4 to specific GAS elements.

An additional way to gain specificity at the DNA level can come from the cooperative interactions that occur among Stat factors as described for the IFN-γ

gene (Xu et al., 1996). Such cooperative binding interactions may enable Stat proteins to recognise variations of consensus sites, promoting selectivity in transcriptional activation. The findings that the construct containing all four IL-12Rβ2 GAS sites not only display an increased responsiveness to IL-12, but maintains Stat4 specificity suggest that also this mechanism is involved in IL-12Rβ2 Stat mediated activation.

Thus, GAS recognition sequences can either bind indiscriminately all Stat, like the IRF-1 GAS sequenced analysed in this chapter, a feature that could account for the overlapping biological effects of some cytokines, or conversely, display a selective recognition pattern like IL-12Rβ2 GAS sites that select among different Stats, activated by different cytokines.

A final point concerns the maintainance of the T helper phenotype, starting from the two following observations. First, Stat4 can still be phosphorylated in response to IFN-α in Th2 cells, yet this does not alter the acquired Th2 phenotype any more (Rogge et al., 1998). Second, Stat4 nuclear localisation is rapidly terminated in the absence of continuos stimulation (Darnell Jr, 1997). These findings strongly suggest that Stat4, while it could potentially initiate IL-12Rβ2 locus remodeling at priming, is unlikely to be involved in maintaining the accessible chromatin structure. Thus, taken together the data presented in the previous chapter and in parallel to the model recently proposed by Agarwal *et al.* for Th2 development (Agarwal and Rao, 1998), it could be hypothesised that Stat4 and antigen activated transcription factors, induced during primary stimulation, activate a differentiation-specific genetic program that results in the recruitment of chromatin remodeling enzymes at the IL-12Rβ2 locus. This would allow yet unidentified transcription factors to bind the locus, contributing to phenotype maintainance. These changes in chromatin configuration will be inherited by subsequent generations of differentiated cells, thus becoming independent of continued Stat4 activation.

3.4) The role of IRF-1 in Th1 development

Very little is known about the molecular mechanisms by which expression of the IFN-γ and IL-4 genes is restricted to the appropriate Th subset. Considerable efforts were made to identify the molecules, and in particular the transcription factors, that are either selectively present or active in developing Th1 or Th2 cells. As discussed in the introduction, the few transcription factors characterised so far are involved in the regulation of the IL-4 gene, while T helper 1 specific transcription factors are even less characterised.

Genetic background of the T cell can influence Th development (see introduction), with some murine strains favoring Th1 development (e.g., B10.D2) and others favoring Th2 development (e.g., BALB/c). It has recently been found that B10.D2 T cells exhibit an intrinsically greater capacity to maintain IL-12 responsiveness under neutral conditions in vitro if compared to BALB/c T cells (Güler et al., 1996; Güler et al., 1997). Analysis of intercrosses between BALB/c and B10.D2 mice identified a locus on murine chromosome 11 that controls the maintenance of IL-12 responsiveness (Gorham et al., 1996).

This chromosomal region is syntenic with a locus on human chromosome 5q31.1 previously shown to be associated with elevated serum IgE levels, suggesting that genetic control of Th1/Th2 differentiation in mouse and of atopy development in humans may be regulated by similar mechanisms (Marsh et al., 1994).

The murine locus contains genes for several cytokines that may influence Th development (see introduction Fig. 2). IL-4 is an obvious candidate, because it directly promotes Th2 development, but T cells and APCs derived from the two strains does not eliminate strain dependent differences in development, suggesting that the underlying genetic mechanisms couldn't be entirely explained through distinct levels of cytokine production. In the same locus localise also transcription factors such as IRF-1 (Miyamoto et al., 1988) and t cell factor (tcf)-1 (Verbeek et al., 1995). IRF-1 may be a good candidate gene in controlling Th1 responses since studies of IRF-1-deficient mice have revealed that this factor is implicated in the

regulation of several immune processes, like T-cell selection and maturation (Penninger et al., 1997; White et al., 1996), development of NK cells (Duncan et al., 1996; Ogasawara et al., 1998; Ohteki et al., 1998), and most importantly, development of Th1 cells (Lohoff et al., 1997; Taki et al., 1997). The compromised Th1 differentiation was associated with defects in the expression of p40 subunit of IL-12 by cells of myeloid origin (Lohoff et al., 1997; Taki et al., 1997). Indeed, a potential IRF-1-responsive element was found in the promoter region of the IL-12 p40 gene (Ma et al., 1996; Murphy et al., 1995).

3.4.1) Regulation of IRF-1 expression in differentiating T helper cells

Two recent studies have demonstrated that IRF-1 deficient mice show a striking defect in the development of Th1 cells (Lohoff et al., 1997; Taki et al., 1997). Interestingly, these two reports differ in their conclusions regarding the relevance of IRF-1 in $CD4^+$ T cells.

To determine the role of IRF-1 on the differentiation of human T helper cells, the expression of IRF-1 was analysed along the differentiation of *naive* T cells under neutral, or Th1 or Th2 polarizing conditions. Purified $CD4^+$, CD45 RO^- T cells isolated from cord blood were stimulated with plate-bound anti-CD3 mAb in the absence of exogenously added cytokines, in the presence of IL-12 and neutralizing anti-IL-4 mAb to induce Th1 development, or in the presence of IL-4 to promote Th2 development. The expression of transcripts encoding IL-12Rβ2 was used to monitor T helper cell differentiation (Fig. 29, lower panel). IRF-1 expression in differentiating Th1 and Th2 cells was analyzed (Fig. 29, upper panel). IRF-1 transcripts were detectable in *naive* $CD4^+$ T cells (lane 1) but were strongly upregulated by TCR-triggering (Fig. 29, upper panel, lane 2). 24 and 48 hours after priming, IRF-1 mRNA was more abundant in cells stimulated in the presence of Th1-inducing conditions than in cells stimulated in the presence of IL-4 or with anti-CD3 mAb alone (lanes 2 and 4). Only a slight downregulation of IRF-1 mRNA is detectable when comparing cultures stimulated in the presence of IL-4 or with

anti-CD3 mAb alone (compare lanes 2, 4 and 5, 7). 96 hours after T cell stimulation, IRF-1 transcripts were clearly more abundant in cultures that had been stimulated in the presence of IL-12.

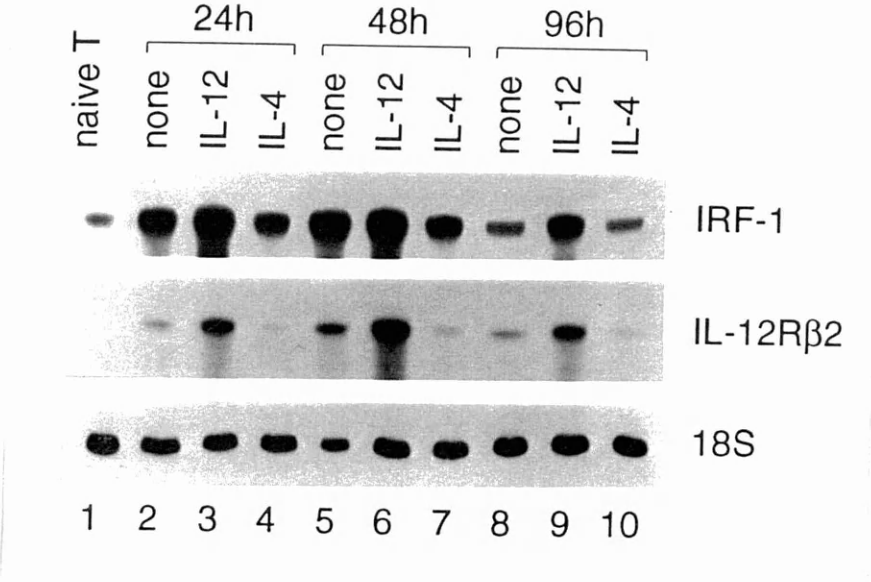

Figure 29. *Antigen receptor triggering induces expression of IRF-1 transcripts in naive CD4⁺ T cells.* $CD4^+$/CD45 RO^- T cells were purified by negative selection from cord blood as described in Materials and Methods. Purified $CD4^+$/CD45 RO^- T were stimulated with plate-bound anti-CD3 mAb with or without the addition of IL-12 (2.5 ng/ml) or IL-4 (1 ng/ml). RNA was extracted from unstimulated cells or at the indicated timeafter CD-3 stimulation. Transcripts encoding IRF-1 (upper panel) and IL-12Rβ2 (lower panel) were quantitated in RNase protection assays.

3.4.2) IRF-1 expression in human Th1 and Th2 cells

Expression of IRF-1 in response to TCR triggering, IL-12 and IL-4 mainly resembles expression of IL-12Rβ2, with two main differences in that IRF-1 transcripts are already present in *naive* T cells and that they are much more

strongly upregulated by TCR activation. The following question was whether IRF-1 transcripts, similarly to IL-12Rβ2 ones, are selectively expressed in differentiated Th1 cells. Thus, expression of IRF-1 transcripts were investigated in Th1 and Th2 lines. RNase protection assays did not reveal significant differences of the IRF-1 mRNA in human Th1 and Th2 cells 2 weeks after stimulation (Fig. 30, lanes 1 and 7).

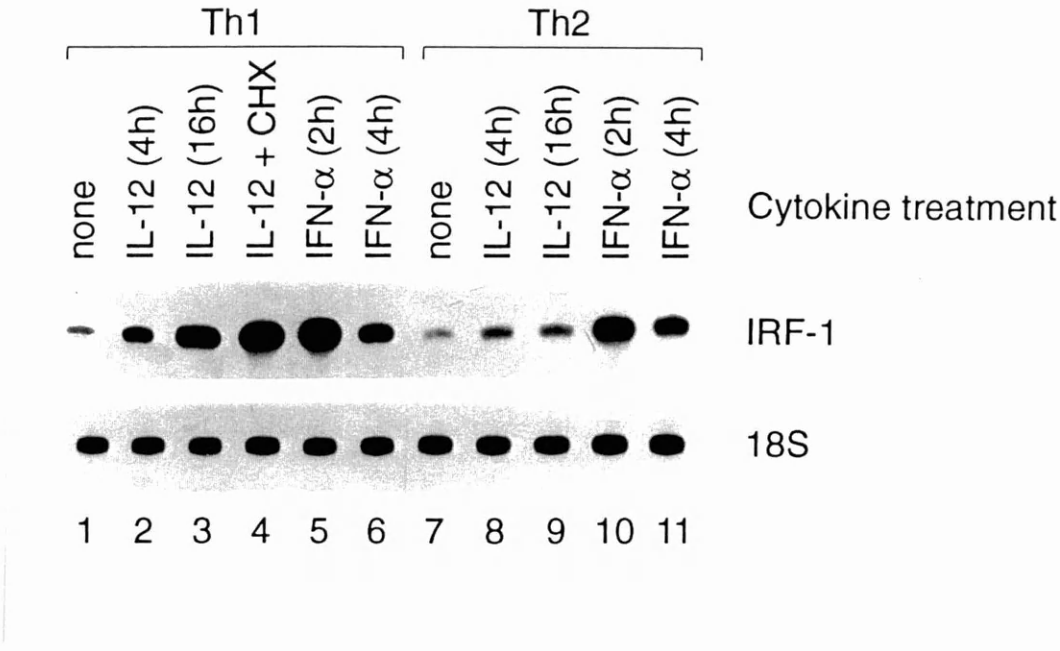

Figure 30. *IL-12 induces IRF-1 transcripts in human Th1 cells.* Th1 and Th2 lines generated from cord blood were harvested 2 weeks after stimulation. The cells were treated as indicated with IL-12 (2.5 ng/ml) or IFN-α (1000 U/ml) followed by the extraction of total RNA. To determined whether de novo protein synthesis is required for IL-12-induced upregulation of IRF-1 transcripts, cells were treated with the protein synthesis inhibitor Cycloheximide (CHX, 10 μg/ml) 45 min prior to the addition of IL-12 (2.5 ng/ml). RNA was extracted 16 h after the addition of IL-12. Transcripts encoding IRF-1 (upper panel) and 18S RNA loading control (lower panel) were analysed by RNase protection assays as described in Materials and Methods.

The effects of IL-12 and IFN-α on IRF-1 mRNA on established Th1 and Th2 lines were also analysed. Interestingly, IRF-1 expression is clearly induced by IL-12 in Th1, but not in Th2 cells (Fig. 30 lanes 2, 3, 8, and 9). The IL-12-induced upregulation of IRF-1 transcripts is not blocked by cycloheximide indicating that *de novo* protein synthesis is not required for IL-2 action (lane 4). IFN-α induced

IRF-1 transcripts in both T helper subsets (lanes 5, 6, 10 and 11). These results evidenced that the IRF-1 gene is not differentially expressed in Th1 in Th1 and Th2 cells, nevertheless these data demonstrate that IRF-1 is a target gene of IL-12 in human Th1 cells.

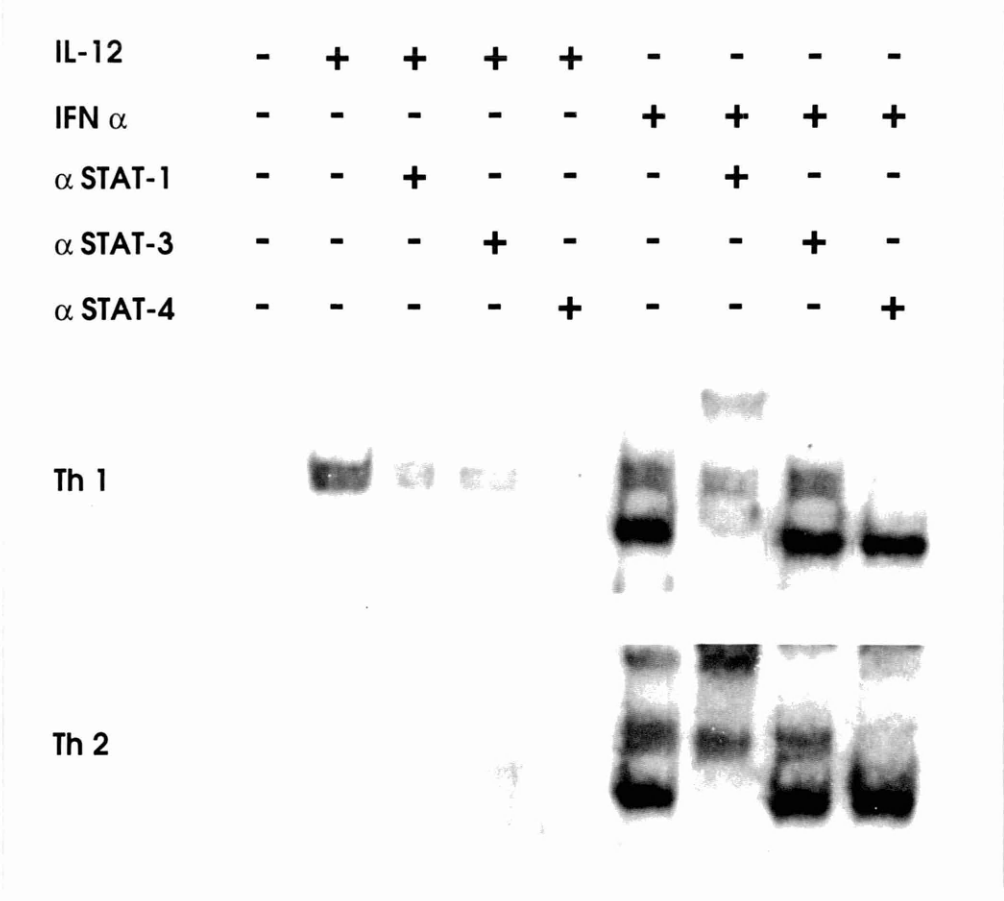

Figure 31. *IL-12 induced binding of Stat4 to the IRF-1 GAS elements in human Th1 cells.* Th1 and Th2 cells were harvested 6 days after priming, washed and resuspended in complete medium. 10^7 cells were incubated at 37 °C in 4 ml complete medium with or without IL-12 (2.5 ng/ml) for 1h or IFN-α (1000 U/ml) for 15 min, followed by preparation of whole cell extracts as described in Material and Methods. Gel shifts were performed with ^{32}P end-labeled double stranded IRF-1 GAS oligonucleotide. Supershift assays were performed using α-Stat1, α-Stat3 and α-Stat4 antibodies as described in Materials and Methods.

The data previously shown utilising the GAS site present in the IRF-1 promoter (see chapters 2 and 3) provided evidence that IL-12 induced expression is mediated by Stat4. Indeed, as shown in figure 31, the IRF-GAS forms DNA-protein complexes in gel shifts performed with IL-12 treated Th1 cells. The complex is

abrogated with Stat4 specific antibodies, while neither anti-Stat1 nor anti-Stat3 antibodies affect the DNA-protein complex.

Furthermore, as shown in figure 32, when the IRF-*luc* construct (that contains 3 copies of the IRFGAS oligonucleotide cloned upstream an SV40-luc reported gene) is transiently transfected in Jurkat cells, a strong upregulation of luciferase activity is detected in response to IL-12. These effects are IL-12Rβ2 and Stat4 dependent, since the absence in the cotransfections of either IL-12Rβ2 or Stat4 DNAs abrogates the IL-12 mediated upregulation of IRF-*luc*.

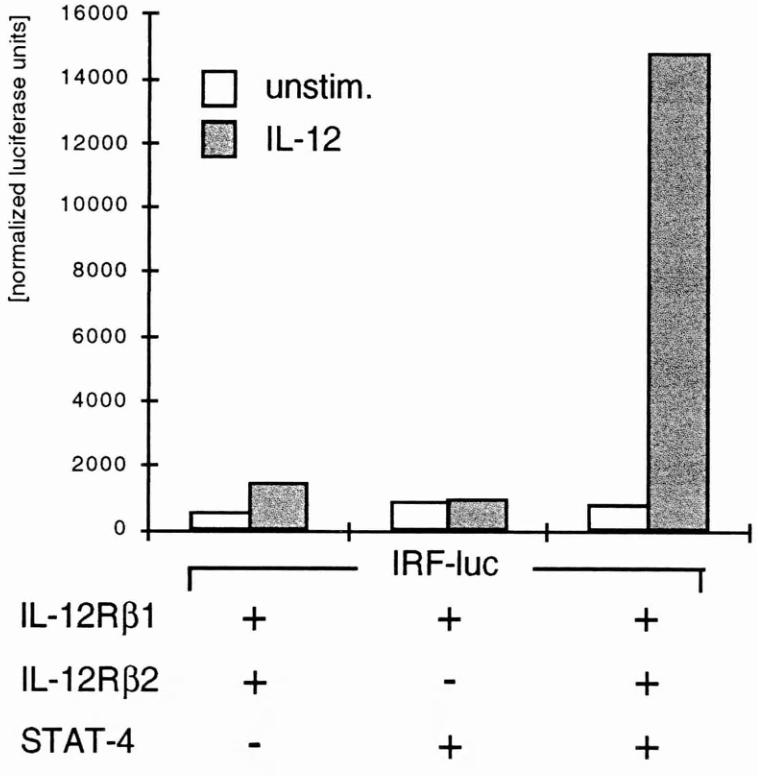

Figure 32. *The IL-12-induced activation of an IRF-1 GAS reporter gene construct is mediated by STAT-4.* Jurkat T cells were transiently transfected with a reporter gene construct containing 3 copies of the GAS element present in the IRF-1 gene promoter. Where indicated, cells were co-transfected with expression vectors encoding IL-12Rβ1, IL-12Rβ2, and/or STAT-4. Cells were left untreated (unstim.) or treated for 20 h with 2.5 ng/ml IL-12 (IL-12) prior to the preparation of lysates. Luciferase assays and normalization of results were performed as described in Experimental Procedures.

To exclude that the upregulation of IRF-1 is secondary to IFN-γ production induced in response to IL-12, the presence of IFN-γ in the surnatants of the transfected cells was investigated by ELISA assays.

There was no detectable IFN-γ production by the transfected cells, indicating that IL-12-dependent induction of luciferase activity is not mediated by IFN-γ (data not shown).

These data provide strong evidence that IL-12 induced activation of the IRF-1 gene is mediated by Stat4 and depends on the presence of functional IL-12 receptors on the cell surface. Moreover, these data suggests that IRF-1 expression in human Th1 cells is regulated by IL-12 through Stat4 activation.

3.4.3) Discussion

In this chapter the role of the transcription factor IRF-1 in the differentiation of human *naive* $CD4^+$ T cells into polarized T helper cell subsets was analysed. Previous results obtained with IRF-1 deficient mice revealed a striking deficiency of Th1 cell development. Compromised Th1 development was shown to be associated with an impaired production of IL-12 by macrophages (Lohoff et al., 1997; Taki et al., 1997) and defective development of natural killer (Duncan et al., 1996; Ogasawara et al., 1998; Ohteki et al., 1998). It remained controversial whether compromised Th1 development also resulted from a defect of $CD4^+$ T cells from IRF-$1^{-/-}$ mice to develop into Th1 effector cells. Cell transfer experiments indicated that IRF-$1^{-/-}$ $CD4^+$ T cells could develop into Th1 cells when transferred to recombination activating gene (RAG)-1 deficient (IRF-$1^{+/+}$) mice, lacking T and B cells (Lohoff et al., 1997). However, the analysis of $CD4^+$ T cells purified from IRF-$1^{-/-}$ TCR transgenic mice showed a deficiency in Th1 development when stimulated *in vitro* with peptide presented by wild-type APCs (Taki et al., 1997).

To determine the role of IRF-1 in the differentiation of T helper subsets, IRF-1 expression was analyzed at different time intervals early after the stimulation of purified *naive* T cells under neutral, or Th1 or Th2-inducing conditions. IRF-1

expression was compared to the expression of the IL-12Rβ2 subunit, which is induced during differentiation of human *naive* cells along the Th1, but not the Th2, pathway. In contrast to IL-12Rβ2, IRF-1 transcripts are detectable in *naive* $CD4^+$ T cells. Crosslinking of the TCR strongly induces IRF-1 mRNA even in the absence of exogenously added cytokines. At later time intervals after T cell stimulation, IRF-1 transcripts are slightly more abundant in cultures which were stimulated in the presence of IL-12.

Analysis of IRF-1 transcripts in established human Th1 and Th2 cells generated from cord blood leukocytes two weeks after stimulation revealed no significant differences in the two populations. However, after treatment with IL-12, IRF-1 transcripts were strongly induced in Th1, but not in Th2 cells. This finding demonstrates that IRF-1 is a target gene of IL-12. The IL-12-induced upregulation of IRF-1 transcripts is not mediated by IL-12 induced IFN-γ, since *i*) IRF-1 mRNA could already be detected after 4 h and did not depend on *de novo* protein synthesis, *ii*) previous results have shown that Th1 cells do not respond to IFN-γ, since they do not express the IFN-γR β subunit (Bach et al., 1995; Groux et al., 1997; Pernis et al., 1995), *iii*) in the transfection experiments IL-12 stimulated Jurkat cells do not produce IFN-γ, but IRF-luc transactivation is strongly increased in response to IL-12.

Previous studies have demonstrated that induction of the IRF-1 gene in response to interferons and IL-6 is mediated by the binding of activated Stat1 and Stat3 to the palindromic GAS element present in the promoter of the IRF-1 gene (Harada et al., 1994; Harroch et al., 1994; Schindler et al., 1995; Sims et al., 1993; Young, 1996).

The data presented in this chapter demonstrate that activated Stat4 is able to transactivate IRF-1 expression in differentiating Th1 cells accounting for a sustained expression of IRF-1. First, gel shift experiments demonstrated that activated Stat4 binds to the GAS element in the IRF-1 promoter. Binding of Stat4 to the IRF-GAS element is induced by IL-12 selectively in Th1 cells and by IFN-α

in both Th1 and Th2 cells, confirming the previous observation that IFN-α signals in human Th1 and Th2 cells (see previous chapter (Rogge et al., 1998)).

Second, transient transfection assays with a reporter gene construct containing 3 copies of the IRF-1 GAS element in Jurkat cells provided strong evidence that the transcriptional activation of the IRF-1 gene in response to IL-12 is mediated by Stat4.

In conclusion, the findings analysed so far point to a potential hierarchy of transcriptional events during the differentiation of *naive* T cells into polarized Th1 cells; TCR-ligation is an essential prerequisite for the initial expression of functional IL-12 receptors on developing Th1 cells, although the mechanisms and factors regulating this phase have not yet been uncovered. In the second phase, IL-12 induces further upregulation of the IL-12Rβ2, IRF-1 and other, yet to be identified, IL-12-regulated genes. The rapid tyrosine phosphorylation and activation of Stat4 by IL-12 and the phenotype of the $Stat4^{-/-}$ mice predict that this phase is regulated by Stat4. In a third phase, transcription factors like IRF-1 and other, so far unknown, regulatory proteins induced by Stat4 may themselves regulate target genes which are important for the effector functions of Th1 cells. IRF-1 appears to be the first member of a probably large family of regulatory proteins induced by IL-12. It will be of great interest to identify additional members of this family and to analyze their functions with respect to the differentiation and effector functions of T helper cells.

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